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(54) Title: HAPTEN MODIFIED TUMOR CELL EXTRACT AND METHODS OF TREATING OR SCREENING FOR CANCER

(57) Abstract

A novel composition comprising a hapten modified tumor cell extract prepared from cancer cells, or cancer cell membranes or peptides obtained therefrom is disclosed. Also disclosed are methods of treating cancer comprising administering therapeutically effective amounts of cyclophosphamide and hapten modified tumor cell extracts, with or without adjuvants and cytokines. Also disclosed is a method of screening for cytokine production by tumors in order to determine the efficacy of treatment, wherein cytokin-specific nucleic acids from a patient tissue sample are amplified in order to produce a detectable signal.

"HAPTEN
MODIFIED TUMOR CELL EXTRACT AND METHODS OF TREATING OR SCREENING
FOR CANCER"

REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of

5 application Serial No. 08/203,004, filed February 28, 1994,

which is a continuation-in-part of application Serial No.

07/985,334, filed December 4, 1992, now U.S. Patent

5,290,551, which is a continuation of application Serial No.

07/520,649, filed May 8, 1990, now abandoned.

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REFERENCE TO GOVERNMENT GRANTS

The invention described herein was made in the

course of work under a grant or award from an NIH Cancer

Research grant, grant no. CA39248. The United States

15 Government may have certain rights in this invention. Some

of this invention was disclosed in a Disclosure Document

filed with the U.S. Patent and Trademark Office on April 18,

1990.

BACKGROUND OF THE INVENTION

It was theorized in the 1960's that tumor cells bear specific antigens (TSA) which are not present on normal cells and that the immune response to these antigens might
5   enable an individual to reject a tumor. It was later suggested that the immune response to TSA could be increased by introducing new immunological determinants on cells. Mitchison, Transplant. Proc., 1970, 2, 92. Such a "helper determinant", which can be a hapten, a protein, a viral coat
10   antigen, a transplantation antigen, or a xenogenous cell antigen, could be introduced into a population of tumor cells. The cells would then be injected into an individual who would be expected to be tolerant to the growth of unmodified tumor cells. Clinically, the hope was that an
15   immunologic reaction would occur against the helper determinants, as a consequence of which the reaction to the accompanying TSA is increased, and tumor cells which would otherwise be tolerated are destroyed. Mitchison, supra, also suggests several modes of action of the helper
20   determinants including 1) that the unmodified cells are merely attenuated, in the sense that their growth rate is slowed down or their susceptibility to immunologic attack increased; 2) that helper determinants merely provide points of attack and so enable the modified cells to be killed by
25   an immune response not directed against TSA; 3) that the helper determinants have an adjuvant action such as binding to an antibody or promoting localization of the cells in the

- 3 -

right part of the body for immunization, in particular, in

lymph nodes.

Fujiwara et al., *J. Immunol.*, 1984a, *132*, 1571

showed that tumor cells conjugated with the hapten,

5   trinitrophenyl (TNP), could induce systemic immunity against

unmodified tumor cells in a murine system, provided that the

mice were first sensitized to the hapten in the absence of

hapten-specific suppressor T cells. Spleen cells from the

treated mice completely and specifically prevented the

10   growth of tumors in untreated recipient animals. Flood et

al., *J. Immunol.*, 1987, *138*, 3573 showed that mice immunized

with a TNP-conjugated, ultraviolet light-induced "regressor"

tumor were able to reject a TNP-conjugated "progressor"

tumor that was otherwise non-immunologic. Moreover, these

15   mice were subsequently resistant to challenge with

unconjugated "progressor" tumor. In another experimental

system, Fujiwara et al., *J. Immunol.*, 1984b, *133*, 510

demonstrated that mice sensitized to trinitrochlorobenzene

(TNCB) after cyclophosphamide pretreatment could be cured of

20   large (10mm) tumors by *in situ* haptenization of tumor cells;

subsequently, these animals were specifically resistant to

challenge with unconjugated tumor cells.

The existence of T cells which cross-react with

ட modified tissues has recently been demonstrated. Weltzien

25   and coworkers have shown that class I MHC-restricted T cell

clones generated from mice immunized with TNP-modified

syngeneic lymphocytes respond to MHC-associated, TNP-

modified "self" peptides. Ortmann, B., et al., *J. Immunol.*,

- 4 -

1992, *148*, 1445. In addition, it has been established that
immunization of mice with TNP-modified lymphocytes results
in the development of splenic T cells that exhibit secondary
proliferative and cytotoxic responses to TNP-modified cells
5   *in vitro*. Shearer, G. M. *Eur. J. Immunol.*, 1974, 4, 527.
The potential of lymphocytes elicited by immunization with
DNP- or TNP-modified autologous cells to respond to
unmodified autologous cells is of considerable interest
because it may be relevant to two clinical problems: I)
10 drug-induced autoimmune disease, and 2) cancer
immunotherapy. In regard to the former, it has been
suggested that ingested drugs act as haptens, which combine
with normal tissue protein forming immunogenic complexes
that are recognized by T cells. Tsutsui, H., et al., *J.*
15 *Immunol.*, 1992, *149*, 706. Subsequently, autoimmune disease,
e.g., systemic lupus erythematosus, can develop and continue
even after withdrawal of absence of the offending drug.
This would imply the eventual generation of T lymphocytes
that cross-react with unmodified tissues.
20   Others have shown that membranes or peptides from
cells, and in one case a peptide from a virus, may elicit a
T cell lymphocyte response *in vitro*. Heike, M., *et al.*, *J*
Immunotherapy, 1994 15:165-174, disclose a method of
stimulating mouse and human antitumor cytotoxic T
25 lymphocytes (CTL) with plasma membrane preparations. This
reference identifies differences among the reports of CTL
responses and associates these differences with differences

- 5 -

in immunogenicity of the tumors used and the mode of
immunization, for example.

Others report that the recognition of human
melanoma cells by cytotoxic T cells is mediated by a number

5   of peptides, Slingluff, C., et al., *J Immunology* 1993
150:2955-2963; Wolfel, T., et al., *Int. J. Cancer* 1994
57:413-418; and Castelli, C., et al., *J. Exp. Med.* 1995
181:363-368, the disclosures of each are incorporated herein
by reference in its entirety. Each of these references

10   indicates that there are a number of T cell defined epitopes
which are unique to a given tumor. None of these references
disclose a tumor cell extract which is obtained by hapten
modification of a tumor cell.

The common denominator of these experiments is

15   sensitization with hapten in a milieu in which suppressor
cells are not induced. Spleen cells from cyclophosphamide
pretreated, TNCB-sensitized mice exhibited radioresistant
"amplified helper function" i.e., they specifically
augmented the in vitro generation of anti-TNP cytotoxicity.

20   Moreover, once these amplified helpers had been activated by
in vitro exposure to TNP-conjugated autologous lymphocytes,
they were able to augment cytotoxicity to unrelated antigens
as well, including tumor antigens (Fujiwara et al., 1984b).
Flood et al., (1987), *supra*, showed that this amplified

25   helper activity was mediated by T cells with the phenotype
Lyt⁻1⁺, Lyt⁻2⁻, L3T4⁺, I⁻J⁺ and suggests that these cells were
contrasuppressor cells, a new class of immunoregulatory T
cell.

- 6 -

Immunotherapy of patients with melanoma has shown

that administration of cyclophosphamide, at high dose (1000

mg/M^2) or low dose (300 mg/M^2), three days before

sensitization with the primary antigen keyhole limpet

5   hemocyanin markedly augments the acquisition of delayed type

hypersensitivity to that antigen (Berd et al., *Cancer Res.*,

1982, *42*, 4862; *Cancer Res.*, 1984a, *44*, 1275). Low dose

cyclophosphamide pretreatment allows patients with

metastatic melanoma to develop delayed type hypersensitivity

10 to autologous melanoma cells in response to injection with

autologous melanoma vaccine (Berd et al., *Cancer Res.*, 1986,

46, 2572). The combination of low dose cyclophosphamide and

vaccine can produce clinically important regression of

metastatic tumor (Berd et al. (1986), *supra*; *Cancer Invest.*,

15 1988a, *6*, 335). Cyclophosphamide administration results in

reduction of peripheral blood lymphocyte non-specific T

suppressor function (Berd et al., *Cancer Res.*, 1984b, *44*,

5439; *Cancer Res.*, 1987, *47*, 3317), possibly by depleting

CD4+, CD45RA+ suppressor inducer T cells (Berd et al.,

20 *Cancer Res.*, 1988b, *48*, 1671). The anti-tumor effects of

this immunotherapy regimen appear to be limited by the

excessively long interval between the initiation of vaccine

administration and the development of delayed type

hypersensitivity to the tumor cells (Berd et al., *Proc.*

25 *Amer. Assoc. Cancer Res.*, 1988c, *29*, 408 (#1626)).

Therefore, there remains a need to increase the therapeutic

efficiency of such a vaccine to make it more immunogenic.

- 7 -

Most tumor immunologists now agree that T
lymphocyte, white cells responsible for tumor immunity,
infiltration into the tumor mass is a prerequisite for tumor
destruction by the immune system. Consequently, a good deal

5   of attention has been focused on what has become known as
"TIL" therapy, as pioneered by Dr. Stephen Rosenberg at NCI.
Dr. Rosenberg and others have extracted from human cancer
metastases the few T lymphocytes that are naturally present
and greatly expanded their numbers by culturing them *in*

10 *vitro* with Interleukin 2 (IL2), a growth factor for T
lymphocytes. Topalian et al., *J. Clin. Oncol.*, 1988, *6*,
839. However this therapy has not been very effective
because the injected T cells are limited in their ability to
"home" to the tumor cite.

15   The ability of high concentrations of IL2 to
induce lymphocytes to become non-specifically cytotoxic
killer cells has been exploited therapeutically in a number
of studies (Lotze et al., *J. Biol. Response*, 1982, *3*, 475;
West et al., *New Engl. J. Med.*, 1987, *316*, 898). However,

20 this approach has been limited by the severe toxicity of
high dose intravenous IL2. Less attention has been given to
the observation that much lower concentrations of IL2 can
act as an immunological adjuvant by inducing the expansion
of antigen activated T cells (Talmadge et al., *Cancer Res.*,

25 1987, *47*, 5725; Meuer et al., *Lancet*, 1989, *1*, 15).
Therefore, there remains a need to understand and attempt to
exploit the use of IL2 as an immunological adjuvant.

- 8 -

Human melanomas are believed to express unique
surface antigens recognizable by T lymphocytes. Old, L. J.,
Cancer Res., 1981, *41*, 361; Van der Bruggen, P., et al.,
Science, 1991, *254*, 1643; Mukherji, B., et al., *J. Immunol.*,

5 1986, *136*, 1888; and Anichini, A., et al., *J. Immunol.*,
1989, *142*, 3692. However, immunotherapeutic approaches to
date have been limited by the difficulty of inducing an
effective T cell-mediated response to such antigens *in vivo*.

There are several models proposed to explicate

10 what appears to be tolerance to human tumor-associated
antigens. They include:

1) Tumor antigen-specific suppressor cells that
down-regulated incipient anti-tumor responses. Mukherji, et
al., *supra*; Berendt, M. J. and R. J. North., *J. Exp. Med.*,

15 1980, *151*, 69.

2) Failure of human tumor cells to elicit T helper
cells or to provide costimulatory signals to those T cells.
Fearon, E. R., et al., *Cell*, 1990, *60*, 397; Townsend, S. E.
and J. P. Allison, *Science*, 1993, *259*, 368; and

20 3) Reduced surface expression of major
histocompatibility products on tumor cells which limits
their recognition by T cells. Ruiter, D. J., *Seminars in
Cancer Biology*, 1991, *2*, 35. None of these hypotheses has
yet been corroborated in a clinical system.

25 The goal of active immunotherapy for tumors is the
development of a productive systemic T cell mediated tumor-
specific immunity. Tumor specific immunity would act both
at the primary tumor site as well as in clearing small

- 9 -

metastatic foci at distant sites. The generation of T cell

immunity has been shown to be a highly regulated response

requiring cell-cell interaction and the production of a

number of cytokines. Of late, studies in a number of human

5   and murine systems have shown that T cell responses can be

separated into two categories termed Type I and II (Mossman,

et al., J. Immunol. 1986 136:2348). Type I responses are

required for the development of delayed type

hypersensitivity (DTH), are associated with macrophage

10 activation and the production of interferon-gamma (IFNγ),

and have been shown to be associated with the resolution of

human leprosy (Yamamura, M., et al., Science 1991 254:277-

279) and murine leishmaniasis (Scott, P., et al.,

Immunological Review 1989 112:161-182). Type II responses

15 are associated with the production of IL4 and IL10,

primarily support antibody responses, and are associated

with the progressive forms of leprosy (Yamamura, M., et al.,

supra) and leishmaniasis (Yamamura et al., supra; and Scott,

P., et al., supra.). In addition to the development of DTH,

20 Type I responses would be expected to enhance the generation

of tumor specific CTL via upregulation of MHC and tumor

associated antigens as well as enhanced antigen presentation

secondary to localized IFNγ production. More recently, Type

I and II responses have been shown to be cross regulating:

25 IFNγ inhibits Type II responses, while IL4 and IL10 inhibit

Type I (Scott, J. Immunol. 1991 147:3149-3155; and

Fiorentino et al., J. Immunol. 1991 146:3444-3451). In the

leishmania system, modulation of cytokines at the lesion

- 10 -

site allows for the conversion of a Type II to a Type I

response, and, consequently, a change from progressive

infection to eradication of the disease (Scott, 1991,

supra).

5   Pisa et al., Proc. Natl. Acad. Sci. USA 1992

89:7708-7712 detected IL10 mRNA in ovarian carcinoma

biopsies, but not in ovarian carcinoma cell lines; they

concluded that the source of IL10 was tumor-infiltrating

lymphocytes. Gastl et al, Int. J. Cancer 1991 55:96-101

10 found that 16/48 tumor cell lines released IL10 into the

culture supernatant; only 3/8 melanoma cell lines were

positive. Finally, Chen et al., Int. J. Cancer, 1994

56:755-760 recently reported that 6/9 cell lines derived

from metastatic melanomas expressed IL10 mRNA. However, the

15 present invention is the first report known to the inventors

of mRNA for IL10 in metastatic melanoma biopsies.

           It is not known whether these observations are

applicable to the human tumor-host relationship, i.e.,

whether the pattern of cytokine production by T cells

20 infiltrating tumors is an indicator of the effectiveness of

the immune response. Patients with metastatic melanoma

treated with an autologous, DNP-modified vaccine develop

inflammatory responses at tumor sites, Berd et al., 1991,

supra. Histologically, these inflamed lesions are

25 characterized by T cell infiltration which is sometimes

associated with tumor cell destruction. The present

invention finds that tumors from DNP-vaccine-treated

patients contain Type I T lymphocytes, which are not

- 11 -

detectable in tumors excised prior to vaccine administration.

SUMMARY OF THE INVENTION

The present invention is directed to a treatment
5   for cancer. Compositions and methods of treating cancer are included in the scope of the present invention. The compositions of the present invention include a composition prepared from a tumor cell or tumor cell extract. The methods of the present invention are directed to treating
10 cancer comprising administering a therapeutically effective amount of a composition comprising a tumor cell or tumor cell extract.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 displays the kinetics of the development
15 of DTH to DNP-modified autologous PBL and melanoma cells. Patients with metastatic melanoma treated with DNP-vaccine were serially skin tested with DNP-modified PBL (LY) or DNP-modified melanoma cells dissociated from a metastatic mass (MEL). Each bar indicates the mean DTH response for the
20 group of patients at each time point; error bars = standard error. For day 119, only responses to PBL were measured. Sample sizes: days 0, 14, 63 N = 84; day 119, N = 57; day 175, N = 42; day 231, N = 35.

Figure 2 exhibits antibody response to DNP. Serum
25 obtained at various time points was tested for antibody (total immunoglobulin) to DNP using an ELISA. The titer was

- 12 -

defined as: (peak OD of sample) X (reciprocal of the
dilution that gave an OD equal to half the peak OD of
positive control).

        Figure 3 is a graph of proliferative response of
5   PBL to DNP-modified autologous lymphocytes. PBL were
obtained from four patients receiving DNP-vaccine at the
peak of their DTH responses. The cells were tested for
ability to proliferate to DNP modified autologous PBL (autol
LY-DNP) with unmodifled autologous PBL (autol LY) as a
10 control. Cultures were pulsed with ^{125}IUDR on day 6.

        Figure 4 shows the kinetics of the proliferative
response to DNP-modified lymphocytes. PBL were serially
collected from patient DM2 while receiving DNP vaccine.
They were cryopreserved and then all samples were tested
15 simultaneously for proliferative response to DNP-modified
autologous PBL (autol LY-DNP). Cultures were pulsed with
^{125}IUDR on day 6.

        Figure 5 displays specificity of the proliferative
response to DNP-modified cells. PBL from two patients were
20 tested for proliferative response to autologous PBL, either
unmodified (autol LY), DNP-modified (autol LY-DNP), or TNP-
modified (autol LY-TNP), and to cultured autologous melanoma
cells, either unmodified (MEL) or DNP-modified (MEL-DNP).
Cultures were pulsed with ^{125}IUDR on day 6.

25   Figure 6 is a specificity analysis of expanded T
cells. PBL from patient DM2 were expanded in IL2 and
repeatedly stimulated with autologous DNP-modified B
lymphoblastoid cells. They were tested for proliferative

- 13 -

response to autologous PBL, either unmodified (autol LY),

DNP modified (autol LY-DNP), or TNP-modified (autol LY-TNP);

cultured autologous melanoma cells, either unmodified (MEL)

or DNP-modified (MELDNP); and allogeneic PBL (Allo LY).

5   Cultures were pulsed with ^{125}IUDR on day 6.

Figure 7 displays responses of CD8+ and CD4+ T

cells to DNP-modified autologous cells. Expanded T cells

were separated into CD8-enriched or CD4-enriched populations

by positive panning. Then they were tested for

10   proliferative response to autologous PBL, either unmodified

(autol LY), DNP-modified (autol LY-DNP), and to cultured

autologous melanoma cells, either unmodified (MEL) or DNP-

modified (MEL-DNP). Cultures were pulsed with ^{125}IUDR on day

6.

15   Figure 8 shows cytokine production by DNP-reactive

T cells. The DNP-reactive T cell line ("Parent"), and three

subcultures (2F8, 1D7, 1C2), obtained by plating at limiting

dilution, were incubated with autologous DNP-modified B

lymphoblastoid cells for 18 hours; supernatants were

20   collected and assayed for gamma interferon (IFN) and IL4.

Figure 9 shows blocking of T cell response by

anti-MHC class I monoclonal antibody. Expanded CD8+ T cells

were stimulated with autologous DNP-modified B

lymphoblastoid cells and the cultures were assayed for gamma

25   interferon after I 8 hours. Stimulator cells were pre-

incubated with one of the following: no antibody (none),

non-specific mouse IgG (non-specific), monoclonal antibody

W6/32 (class I), or monoclonal antibody L243 (class II).

- 14 -

Figure 10 exhibits MHC restriction of T cell
response. Expanded CD8+ T cells (HLA-AI, A2, B8, Bw6) were
tested for ability to proliferate in response to DNP-
modified autologous PBL and to DNP modified allogeneic PBL
5 from four other patients. Three of the allogeneic
stimulators were matched at one or more class I loci as
shown, and the fourth was completely mismatched (A24, A26,
B44, B63). Cultures were pulsed with ^{125}IUDR on day 6.

Figure 11 shows graphs of cytotoxicity of DNP-
10 reactive T cells. Melanoma cells, either autologous (autol)
or allogeneic class I-mismatched (allo), were used as
targets in a 6-hour ^{51}Cr assay. Effector cells were expanded
CD8+, DNP-reactive T cells. Figure 11A - target cells were
haptenized with various concentrations of DNBS or TNBS. The
15 effector: target cell ratio was 20:1. Figure 11B - Target
cells haptenized with 2.5 mg/ml DNBS or TNBS were mixed with
effector cells at a series of effector: target (E:T) ratios.

Figure 12 displays a graph of the percentage of
patients tumor free in the months following surgery treated
20 with DNP vaccine and non-haptenized control vaccine.

Figure 13 shows the HPLC fractions which were
pooled into five groups of ten fractions each. Peptides
derived from dinitrophenyl modified melanoma cells (DNP-MEL)
or dinitrophenyl modified B cells (DNP-LY) were stimulatory
25 in pool 2.

Figure 14 displays an inflamed subcutaneous
melanoma nodule from DNP-vaccine immunized patient expresses
mRNA for IFNγ and IL10. Figure 14-1 shows mRNA for

- 15 -

cytokines determined by RT-PCR (lane 1 = size marker; 2 = β-actin; 3 = IFNγ, 4 = IL4; 5 = IL10); Figure 14-2 is an H&E stained section of the subcutaneous lesion (400X).

Figure 15 exhibits a lymph node metastasis from an
5 unimmunized patient expresses mRNA for IL10 but not IFNγ. Figure 15-1 shows mRNA for cytokines determined by RT-PCR (lane 1 = size marker; 2 = β-actin; 3 = IFNγ; 4 = IL4; 5 = IL10); Figure 15-2 is an H&E stained section of the lymph node metastasis (400X).

10   Figure 16 is a gel of IL10 mRNA expressed in human melanoma metastases. mRNA for cytokines was determined by RT-PCR (lane 1 = size marker; C = IL10 cDNA control; 1-7 = patient samples).

Figure 17 is a gel of IL10 mRNA expressed by the
15 melanoma cells. Figure 17 shows IL10 mRNA expression by RT-PCR from representative tumor biopsy and derived cell line (lane 1 = size marker; 2 = IL10 cDNA; 3 = tumor biopsy; 4 = tumor line).

Figure 18 is an *in-situ* RT-PCR from a paraffin
20 section of a non-inflamed melanoma biopsy (A=100x, B=400x).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to cancer immunotherapy. A novel tumor composition and methods of treating cancer are included in the scope of the invention.

25   The present invention is directed for use in treating cancer, including metastatic and primary cancers. Cancers treatable with the present invention include the

- 16 -

following non-limiting examples: melanoma, breast, lung,
colon, breast, kidney, and prostate. Mammals, particularly
humans, having metastatic cancer may be treated with the
compositions and methods of the present invention.

5   The compositions of the present invention are
prepared from a tumor cell or tumor cell extract. A tumor
cell may be a malignant or pre-malignant cell of any type of
cancer. In accordance with the present invention, pre-
malignant refers to any abnormal cell suggestive of a cancer
10 cell, which is not yet a cancer cell; such as and not
limited to displastic changes in cervical cells which
ultimately lead to cervical cancer, and displastic nevi
which are abnormal skin cells which lead to melanoma. The
tumor cells and extracts preferably originate from the type
15 of cancer which is to be treated. The tumor cells and
extracts may be, and are not limited to, autologous and
allogenic cells dissociated from biopsy specimens or tissue
culture, as well as stem cells and extracts from these
sources. Preferably, the cells and extracts are autologous.
20 Tumor cell extracts of the present invention may be a
peptide isolated from a hapten modified cancer cell or a
cell membrane isolated from a hapten modified cancer cell.

           For purposes of the present invention, peptides
25 are compounds of two or more amino acids and includes
proteins. Peptides will preferably be of low molecular
weight, of about 1,000 kD to about 10,000 kD, more
preferably about 1,000 to about 5,000, which are isolated

- 17 -

from a haptenized tumor cell and which stimulate T cell

lymphocytes to produce gamma interferon. T cells are

lymphocytes which mediate two types of immunologic

functions, effector and regulatory, secrete proteins

5   (lymphokines), and kill other cells (cytotoxicity).

Effector functions include reactivity such as delayed

hypersensitivity, allograft rejection, tumor immunity, and

graft-versus-host reactivity. Lymphokine production and

cytotoxicity are demonstrated by T cell effector functions.

10 Regulatory functions of T cells are represented by their

ability to amplify cell-mediated cytotoxicity by other T

cells and immunoglobulin production by B cells. The

regulatory functions also require production of lymphokines.

T cells produce gamma interferon (IFNγ) in response to an

15 inducing stimulus including and not limited to mitogens,

antigens, or lectins. The peptide may preferably be about 8

to about 20 amino acids, in addition the peptide is

preferably be haptenized. Peptides may be isolated from the

cell surface, cell interior, or any combination of the two

20 locations. The extract may be particular to type of cancer

cell (versus normal cell). The peptide of the present

invention includes and is not limited to a peptide which

binds to the major histocompatibility complex, a cell

surface-associated protein, a protein encoded by cancer

25 oncogenes or mutated anti-oncogenes.

The cancer cell membrane of the present invention

may be all or part of a membrane from a membrane isolated

from a haptenized cancer cell. In accordance with the

- 18 -

definition of cancer cell membrane as set forth for the

present invention, a cancer cell membrane may be isolated

then haptenized, alternatively, a cancer cell may be

haptenized and the membrane subsequently isolated therefrom.

5   The cell extracts are able to stimulate T cells.

Stimulation for purposes of the present invention refers to

proliferation of T cells as well as production of cytokines

by T cells, in response to the cell extract. Membranes and

proteins isolated from hapten modified tumor cells and

10 proteins each independently have the ability to stimulate T

cells. Proliferation of T cells may be observed by uptake

by T cells of modified nucleic acids, such as and not

limited to ^{3}H thymidine, ^{125}IUDR (iododeoxyuridine); and dyes

such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

15 bromide (MTT) which stains live cells. In addition,

production of cytokines such as and not limited to IFNγ,

tumor necrosis factor (TNF), and IL-2. Production of

cytokines is preferably in an amount of greater than 15

picograms/ml, more preferably about 20 to about 30

20 picograms/ml, even more preferably about 50 picograms/ml.

        Preferably, the tumor cell extract comprises

cellular materials which are unique, or substantially

specific to, a particular type of cancer. The tumor cells

of the present invention may be live cells. The tumor cells

25 and extracts of the present invention may be irradiated

prior to use. Tumor cells or extracts are irradiated at

about 2500 cGy to prevent the cells from growing after

injection.

- 19 -

The compositions of the invention may be employed
in the method of the invention singly or in combination with
other compounds, including and not limited to other
compositions of the invention. Accordingly, cancer cells

5   and cancer cell extracts may be used alone or co-
administered. For purposes of the present invention, co-
administration includes administration together and
consecutively. In addition, the cancer cell membrane may be
co-administered with the peptide. Further, the cancer cells

10 and/or extracts may be co-administered with other compounds
including and not limited to cytokines such as interleukin-
2, interleukin-4, gamma interferon, interleukin-12, GM-CSF.
The tumor cells and extracts of the invention may also be
used in conjunction with other cancer treatments including

15 and not limited to chemotherapy, radiation, antibodies,
oligonucleotide sequences, and antisense oligonucleotide
sequences.

The compositions of the invention may be
administered in a mixture with a pharmaceutically-acceptable

20 carrier, selected with regard to the intended route of
administration and the standard pharmaceutical practice.
Dosages may be set with regard to weight, and clinical
condition of the patient. The proportional ratio of active
ingredient to carrier naturally depend on the chemical

25 nature, solubility, and stability of the compositions, as
well as the dosage contemplated. Amounts of the tumor cells
and extracts of the invention to be used depend on such
factors as the affinity of the compound for cancerous cells,

the amount of cancerous cells present and the solubility of
the composition. The compounds of the present invention may
be administered by any suitable route, including inoculation
and injection, for example, intradermal, intravenous,

5   intraperitoneal, intramuscular, and subcutaneous.

        The compositions of the present invention may be
administered alone or will generally be administered in
admixture with a pharmaceutical carrier selected with regard
to the intended route of administration and standard

10 pharmaceutical practice.

        The composition of the present invention is a
therapeutically effective amount of a composition selected
from the group consisting of live tumor cells, tumor cell
extracts, such as a peptide and a cancer cell membrane, and

15 a mixture of tumor cells and one or more tumor cell
extracts. The composition may be mixed with an
immunological adjuvant and/or a pharmaceutically acceptable
carrier. Any known aqueous vehicle useful in drug delivery,
such as and not limited to saline, may be used in accordance

20 with the present invention as a carrier. In addition, any
adjuvant known to skilled artisans may be useful in the
delivery of the present invention. Adjuvants include and
are not limited to *Bacillus Calmette-Guerin (BCG)*;
cytokines, such as and not limited to interleukin-12,

25 interleukin-2; and synthetic adjuvants such as and not
limited to QS-21, (Cambridge Biotech, Worcester, MA)
disclosed by Livingston *et al.*, *Vaccine* 1994 *12*:1275, the
disclosure of which is incorporated herein by reference in

- 21 -

its entirety. In the case where the cells and cell extracts
are irradiated and haptenized, the cells may be conjugated
to a hapten and then irradiated. Alternatively, the cells
may be irradiated then conjugated to a hapten. In either

5 case, the extracts are subsequently purified and then may
also be irradiated and/or haptenized. To irradiate and
haptenize the extracts, either method may be performed
first, followed by the other method.

Alternatively, the tumor cells or tumor cell

10 extracts may be added to antigen presenting cells. The
cancer cell extract may be used to treat cancer together
with another cell type, an antigen presenting cell, selected
from the group consisting of autologous cultured macrophages
and autologous cultured dendritic cells. Macrophages are

15 any large ameboid mononuclear cell, regardless of origin,
such as and not limited to histiocytes and monocytes, which
phagocytose, i.e. engulf and destroy, other cells, dead
tissue, degenerated cells, and the like. Macrophages are
antigen presenting cells, which present antigens, including

20 tumor antigens, to cells including T cells. Dendritic cells
are also antigen presenting cells and appear to be closely
related to macrophages, however, dendritic cells are more
efficient antigen presenting cells than macrophages. They
are potent stimulators of T cells and may be isolated from a

25 variety of body organs and tissues including and not limited
to blood, skin (where dendritic cells are referred to as
Langerhans cells), lymphoid tissues.

The antigen presenting cells with peptide or
membrane bound thereto, for example, may be used to immunize
patients. The patient's blood is obtained and macrophages
or dendritic cells are extracted therefrom. High

5   concentrations of the peptide (about 1 ng/ml to about 1
μg/ml, preferably about 10 ng/ml to about 100 ng/ml), or
membrane (about 10^5 to about 10^7 cell equivalents, cell
equivalents are in relation to the number of starting cells)
are incubated with the cells overnight or for about 8 hours.

10 In the case of incubating with membranes, the membranes are
phagocytized by the macrophages or dendritic cells. The
macrophages or dendritic cells which have phagocytized the
membranes are used to immunize the patient, Grabbe, S., et
al., Immunology Today 1995 16:117-121, the disclosure of

15 which is incorporated herein by reference in its entirety.

In a preferred embodiment of the invention, the
composition comprises a vaccine consisting of about 10 x 10^6
to about 25 x 10^6, more preferably about 5 x 10^6 to about 25
x 10^6, live, irradiated, tumor cells suspended in a

20 pharmaceutically acceptable carrier or diluent, such as and
not limited to Hanks solution, saline, phosphate-buffered
saline, and water, to which is added an immunological
adjuvant, such as and not limited to Bacillus Calmette-
Guerin (BCG). The tumor cells and extracts may be

25 conjugated to a hapten. The mixture is injected
intradermally into 3 contiguous sites per administration on
the upper arms or legs, excluding limbs ipsilateral to a
lymph node dissection.

- 23 -

Patients may be immunized to the chemical
dinitrophenyl (DNP) by application of dinitrofluorobenzene
(DNFB) to the skin. Two weeks later, the patients are
injected with a vaccine, which may include irradiated cells
5 haptenized to DNP. The vaccine is reinjected every 4 weeks
for a total of eight treatments. The drug cyclophosphamide
(CY) may be administered 3 days prior to each vaccine
administration to augment the immune response to the tumor
cells. A non-haptenized form of the vaccine may be
10 similarly administered.

The vaccine of the present invention may be
haptenized or non-haptenized. The haptenized, or
chemically-linked, form of the vaccine may include a tumor
cell haptenized to dinitrophenyl (DNP) for example. Other
15 haptens include and are not limited to trinitrophenyl (TNP)
and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine
(AED). A vaccine of tumor cell extracts may similarly be
haptenized. In the case of haptenized cancer cell extracts,
the extracts, a peptide, and a cancer cell membrane, are
20 isolated from haptenized cancer cells. The present
invention also contemplates a non-haptenized vaccine of
tumor cells and/or cell extracts.

In the methods of the present invention, a method
of treating a patient suspected of having cancer, comprises
25 administering a pharmaceutically acceptable amount of
cyclophosphamide, a pharmaceutically acceptable amount of a
composition selected from the group consisting of live tumor
cells, tumor cell extracts, and a mixture of tumor cells and

- 24 -

tumor cell extracts. Where the composition is a cancer cell

extract, the extract may be a peptide or a membrane isolated

from a haptenized cancer cell. The composition may be mixed

with an immunological adjuvant and/or a pharmaceutically

5   acceptable carrier. The haptenized vaccine may optionally

be followed by administration of a pharmaceutically

acceptable amount of a non-haptenized vaccine. A non-

haptenized vaccine may also be administered in accordance

with the methods of the present invention.

10   The vaccine of the present invention may comprise

tumor cells and/or tumor cell extracts. The tumor cells for

use in the present invention may be prepared as follows.

Tumor masses are processed as described by Berd et al.

(1986), *supra*, incorporated herein by reference in its

15   entirety. The cells are extracted by enzymatic dissociation

with collagenase and DNAse by mechanical dissociation,

frozen in a controlled rate freezer, and stored in liquid

nitrogen until needed. On the day that a patient is to be

skin tested or treated, the cells are thawed, washed, and

20   irradiated to about 2500 R. They are washed again and then

suspended in Hanks balanced salt solution without phenol

red. Conjugation of the prepared cells with DNP is

performed by the method of Miller and Claman, *J. Immunol.*,

1976, *117*, 1519, incorporated herein by reference in its

25   entirety, which involves a 30 minute incubation of tumor

cells with DNFB under sterile conditions, followed by

washing with sterile saline.

Cancer cells of a patient may be conjugated to a
hapten by isolating the membranes and modifying the
membranes or by conjugating the cells to a hapten without
first isolating the membranes.

5   A cancer cell membrane may be prepared by
isolating membranes from non-modified preparation of cancer
cells of a patient. Cells are suspended in about five
volumes of about 30 mM sodium bicarbonate buffer with about
1 mM phenyl methyl sulfonyl fluoride and disrupted with a
10 glass homogenizer. Residual intact cells and nuclei are
removed by centrifugation at about 1000 g. The membranes
are pelleted by centrifugation at 100,000 g for 90 minutes.
The membranes are resuspended in about 8% sucrose and frozen
at about -80°C until needed. To a suspension of membranes
15 (about 5,000,000 cell equivalents/ml), about 0.5 ml of 1
mg/ml dinitrofluorobenzene (DNFB) is added for about 30
minutes. Similarly, other haptens such as and not limited
to trinitrophenyl and N-iodoacetyl-N'-(5 sulfonic 1-naphtyl)
ethylene diamine may be used. Excess DNP is removed by
20 dialyzing the membranes against about 0.15 M PBS for about
three days. The membranes are pelleted.

          Alternatively, the cancer cell extract, the
peptide or the membrane, may be prepared by modifying cancer
cells of a patient with a hapten such as dinitrophenyl and
25 then preparing membranes therefrom. Cancer cells of a
patient are obtained during biopsy and frozen until needed.
About 100 mg of DNFB (Sigma Chemical Co., St. Louis, MO) was
dissolved in about 0.5 ml of 70% ethanol. About 99.5 ml of

- 26 -

PBS was added. DNFB concentration should be about 152

mg/0.1 ml. The solution was stirred overnight in a 37 °C

water bath. The shelf life of the solution is about 4

weeks. The cells were thawed and the pellet was resuspended

5   in 5X cells/ml in Hanks balanced salt solution. About 0.1

ml DNFB solution was added to each ml of cells and incubated

for about 30 minutes at room temperature. Similarly, other

haptens such as and not limited to trinitrophenyl and N-

iodoacetyl-N'-(5 sulfonic 1-naphtyl) ethylene diamine may be

10   used. The cells were then washed twice in Hanks balanced

salt solution. Cells are suspended in about five volumes of

about 30 mM sodium bicarbonate buffer with about 1 mM phenyl

methyl sulfonyl fluoride and disrupted with a glass

homogenizer. Residual intact cells and nuclei are removed

15   by centrifugation at about 1000 g. The membranes are

pelleted by centrifugation at 100,000 g for 90 minutes. The

membranes are resuspended in about 8% sucrose and frozen at

about -80°C until needed.

From the DNP modified cells, peptide may be

20   extracted, some of which are DNP modified as a result of

modifying the cells. Protein extraction techniques, known

to those of skill in the art, may be followed by antigen

assays to isolate antigen(s) effective for patient

treatment. The methods of isolating cell extracts are

25   readily known to those skilled in the art. Briefly,

cancerous cells are isolated from a tumor and cultured *in*

vitro. Dinitrophenyl is added to the cultured cells in

accordance with the method set forth above. Peptides are

- 27 -

isolated from cells according to an established technique of
Rotzschke et al., *Nature*, 1990, *348*, 252, the disclosure of
which is hereby incorporated by reference in its entirety.
The cells are treated with a weak acid. Then they are
5   centrifuged and the supernatants are saved. Fractions
containing small peptides are obtained by HPLC,
concentrated, and frozen. The fractions are screened for
immunological activity by allowing them to bind to
autologous B lymphoblastoid cells which are then tested for
10 ability to stimulate melanoma-specific T lymphocytes.

        The cells are treated with a weak acid, such as
and not limited to trifluoroacetic acid (TFA). The cells
are then centrifuged and the supernatant is saved.
Compounds having a molecular weight greater than 5,000 were
15 removed from the supernatant by gel filtration (G25
Sepharose, Pharmacia). The remainder of the supernatant is
separated on a reversed-phase HPLC column (Superpac Pep S,
Pharmacia LKB) in 0.1% trifluoroacetic acid (TFA) using a
gradient of increasing acetonitrile concentration; flow rate
20 = 1 ml/min, fraction size = 1 ml. Fractions containing
small peptides are obtained by HPLC according to the method
of Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*,
2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring
Harbor, NY (1989), concentrated, and frozen. The fractions
25 are screened for immunological activity by allowing them to
bind to autologous B lymphoblastoid cells which are then
tested for ability to stimulate melanoma-specific T
lymphocytes.

- 28 -

Epstein barr virus (EBV, ATCC CRL-1612, B95-8 EBV

transformed leukocytes, cotton top marmoset, *Saguinus*

oedipus) is added to B lymphoblastoid cells in culture. The

B lymphoblastoid cells are transformed into a B cell tumor

5   from the patient's own lymphocytes. Melanoma from a

metastasis is cultured in RPMI 1640 + 10% fetal calf serum

or 10% pooled human serum. The non-adhered cells are washed

off with RPMI medium. When the cells are confluent, they

are detached with 0.1% EDTA and split into two flasks. This

10 process continues where the confluent cells are continuously

split. To test for gamma interferon production by T cells,

lymphocytes from a patient's blood are obtained. The

patient's own tumor cells, which have been modified with a

hapten, such as DNP, are mixed with the lymphocytes to

15 stimulate the T cells. Every seven days, interleukin-2 is

added. The T cells are expanded by splitting a as disclosed

above. The T cells are then restimulated by the hapten

modified cells. An enriched population of T cells result

which are responsive to the hapten modified cells.

20   Human cancer vaccines have been developed and

tested by a number of workers. Although they can sometimes

induce weak immunity to a patient's cancer, they rarely

cause tumor regression. The development of inflammatory

responses in metastatic tumors was surprisingly found with

25 the DNP-vaccine of the present invention. The tumor becomes

reddened, warm and tender. Ultimately, in some cases, the

tumor regresses to the extent that the tumor disappears, to

the naked eye and microscopically. Microscopically,

- 29 -

infiltration of T lymphocytes into the tumor mass is

observed. Therefore, this approach, which increases the

inflammatory response and the number and capacity of

lymphocytes entering the tumor, is a significant advance in

5 the art.

The effectiveness of the vaccine may be improved

by adding various biological response modifiers. These

agents work by directly or indirectly stimulating the immune

response. Biological response modifiers of the present

10 invention include and are not limited to interleukin-12 and

gamma interferon. In this embodiment, IL12 will be given

following the each vaccine injection. Administration of

IL12 to patients with inflammatory responses is believed to

cause the T lymphocytes within the tumor mass to proliferate

15 and become more active. The increased T cell numbers and

functional capacity leads to immunological destruction of

the tumors. Dosages for IL12 will be prepared in accordance

with the dosage indications set forth above.

Patients with metastatic melanoma were treated

20 using an immunotherapy regimen with the following

components: 1) vaccine consisting of autologous tumor cells

conjugated to DNP; and 2) low dose cyclophosphamide

pretreatment. Patients were evaluated to determine whether

tumor regression had occurred, to monitor tumor inflammatory

25 responses, and to measure delayed type hypersensitivity to

autologous melanoma cells, DNFB (the form of DNP used for

skin sensitization), DNP-conjugated autologous lymphocytes,

diluent (Hanks solution), purified protein derivative (PPD),

and recall antigens (candida, trichophyton, and mumps).
Patients who are considered to be deriving benefit (clinical
or immunological) from the therapy are continued in the
immunotherapy regimen. Subsequent vaccines may be given
5 without cyclophosphamide. In a similar experiment,
Interleukin 2 linked to polyethylene glycol was found to not
be effective.

In another embodiment, a vaccine comprising
chemical extracts of cancer cells conjugated to a hapten and
10 mixed with an immunological adjuvant, such as *Bacillus
Calmette-Guerin*, BCG, is used.

In the present invention, biopsies from human
melanoma metastases were examined for expression of cytokine
mRNA using RT-PCR. mRNA for IFNγ is found in post-DNP-
15 vaccine, inflamed metastases, but only rarely in
pretreatment metastases, even those containing large numbers
of residual lymph node lymphocytes. Moreover, the Type II
cytokine, IL10, is found in almost all melanoma metastases
and appears to be produced by the melanoma cells themselves.
20 Patients with metastatic melanoma treated with an
autologous, DNP-modified vaccine develop inflammatory
responses at tumor sites. Histologically, these inflamed
lesions are characterized by T cell infiltration which is
sometimes associated with tumor cell destruction. In the
25 present invention, biopsy specimens of 8 subcutaneous
metastases that had developed inflammation following vaccine
treatment were tested for expression of mRNA for IFNγ, IL4,
TNF, and IL10. Post-vaccine, inflamed biopsies contained

- 31 -

mRNA for IFNγ (5/8), IL4 (4/8) or both (3/8), and for TNF

(4/7). In contrast, IFNγ mRNA was detected in only 1/17 and

TNF mRNA in 2/16 control specimens (pre-treatment lymph node

metastases or non-inflamed subcutaneous metastases). mRNA

5 for IL10, a cytokine with anti-inflammatory properties, was

detected in 24/25 melanoma metastases and was independent of

lymphoid content; in situ PCR confirmed that melanoma cells

were the major source. These findings provide a new

parameter by which to measure the effects of cancer

10 immunotherapy.

The present invention is aimed at analyzing

freshly obtained metastatic melanoma biopsies for the

presence of cytokine mRNA which correlates with a productive

immune response at the tumor site. The expression of IFNγ

15 or IL4 mRNA is characteristic of melanoma metastases that

have developed an inflammatory response following

administration of DNP-modified autologous vaccine. On the

other hand, expression of IL10 mRNA is independent of an

inflammatory response and seen in nearly all melanoma biopsy

20 specimens. Examination of cell lines derived from melanoma

biopsies as well as in situ PCR analysis demonstrated that

the source of IL10 is melanoma cells themselves rather than

the associated lymphocytes.

Perhaps the most important finding of this work is

25 the negative one: mRNA for IFNγ and IL4 generally is not

found in melanoma metastases from untreated patients, nor in

metastatic masses that contain large numbers of lymph node

lymphocytes. This provides a low background activity of in

situ cytokine production against which to compete melanoma tissues whose T cell population has been altered by immunotherapy. Moreover, it underscores an important biological point: T cells extracted from melanoma nodal

5 metastases probably represent the residua of the original lymph node population rather than lymphocytes that have actually infiltrated the tumor as a result of their recognition of melanoma antigens. Since they are not antigen-activated, they have received no stimulus to produce

10 IFNγ or IL4.

In contrast, biopsy specimens obtained following administration of DNP-vaccine typically expressed mRNA for IFNγ. However, DNP-vaccine-induced inflammatory responses cannot be characterized as Type I since some of these

15 samples contained IL4 as well. Given the sensitivity of PCR-based mRNA analysis, such a pattern could be produced by a small focus of IL4-producing T cells in the midst of a T cell infiltrate that is predominantly IFNγ-producing. On the other hand, the presence of mRNA for IFNγ and IL4 could

20 signify the presence of T cells that produce both cytokines - so-called TH$_0$ cells (Lee et al, *Eur. J. Immunol.* 1992 *22*:1455-1459). Resolution of this issue will require analyses that allow correlation of mRNA expression with morphology, such as in situ PCR. Whatever the results,

25 these findings suggest that measured of intra-tumor cytokine production may be an important parameter to measure in patients undergoing immunotherapy.

The present invention strongly suggest that the
source of IL10 mRNA is the melanoma cells themselves, rather
than the associated lymphocytes. Strong IL10 mRNA bands
were detected in 24/25 biopsies, and its expression was

5   independent of the number of associated lymphocytes or the
presence of DNP-vaccine-induced inflammation. Moreover, in
situ PCR clearly showed IL10 mRNA within melanoma cells.
Cell lines derived from the biopsy material expressed IL10
mRNA and produced IL10 as measured by ELISA.

10   The physiologic significance of IL10 production in
melanoma tissues is not clear. IL10 is known to be an anti-
inflammatory cytokine with ability to inhibit T cell
proliferation and IL2 production (Jinquan, T., et al., J.
Immunol. 1993 151:4545-4551) and delayed type

15 hypersensitivity (Lee, supra), probably by reducing
macrophage costimulatory function. Thus IL10 could suppress
the activation and proliferation of melanoma-reactive T
cells that have infiltrated the tumor site. However, IL10
recently has been shown to be a chemoattractant for CD8+ T

20 cells (Jinquan, supra); this property could account for the
predominance of CD8+ cells in DNP-vaccine-induced lymphoid
infiltrates. In either case, modulation of IL10 production
at the tumor site may have important consequences for the
tumor-host relationship.

25   The scope of the present invention also includes a
method of screening for cytokine production by a tumor to
determine the efficacy of an autologous, irradiated hapten
conjugated cell composition in a patient suspected of having

- 34 -

cancer, said method comprising administering said hapten

conjugated composition to said patient; obtaining a sample

comprising nucleic acids from a patient tissue sample;

amplifying nucleic acids specific for a cytokine or

5   amplifying a signal generated by hybridization of a probe

specific to a cytokine specific nucleic acid in said tissue

sample; and detecting the presence of the amplified nucleic

acids or the amplified signal wherein the presence of

amplified nucleic acids or amplified signal indicates

10   cancer, wherein the presence of amplified nucleic acids or

amplified signal from said patient tissue sample indicates

efficacy of said hapten conjugated composition.

The tissue sample may be a malignant or pre-

malignant tumor, a melanoma tumor for example, or a

15   subcutaneous inflammatory metastatic melanoma, for example.

In addition, a tissue sample may be a solid or liquid tissue

sample such as and not limited to all or part of a tumor,

saliva, sputum, mucus, bone marrow, serum, blood, urine,

lymph, or a tear from a patient suspected of having cancer.

20   Nucleic acids, such as DNA (including cDNA) and

RNA (including mRNA), are obtained from the patient tissue

sample. Preferably RNA is obtained from a tissue sample.

Total RNA is extracted by any method known in the art such

as described in Sambrook et al., *Molecular Cloning: A*

25   *Laboratory Manual* (Cold Spring Harbor Laboratory, Cold

Spring Harbor, NY, 1989), incorporated herein by reference

in its entirety.

- 35 -

Nucleic acid extraction is followed by
amplification of the same by any technique known in the art.
The amplification step incudes the use of at least one
primer sequence which is complementary to a portion of a
5   cytokine specific sequence. Cytokine specific sequences are
defined for purposes of the present invention to include
(and are not limited to) all or part of sequences which
encode IFNγ, TNF, IL-2, IL-12, and IL-13. Generally, the
primer sequence is about 21 nucleotides to about 33
10 nucleotides, preferably about 21 nucleotides, about 31
nucleotides, 32 nucleotides, and about 33 nucleotides in
length.

Primer sequences useful in the amplification
methods include and are not limited to β actin, SEQ ID NOS:
15 1 and 2; IFNγ, SEQ ID NOS: 3 and 4; IL4, SEQ ID NOS: 5 and
6; IL10, SEQ ID NOS: 7 and 8; and TNF, SEQ ID NOS: 9 and 10.

Where a template dependent process of
amplification uses a pair of primers, one primer of the pair
may comprise oligonucleotides which are complementary to
20 nucleic acid sequences which encode cytokine specific
proteins. The one primer of the pair may be selected from
the group consisting of SEQ ID NOS: 1 to 10.

Alternatively, each of the two oligonucleotides in
the primer pair may be specific to a nucleic acid sequence
25 which encodes a cytokine. The primers may be designed to be
complementary to separate regions of a cytokine sequence for
example. By separate regions is meant that a first primer
is complementary to a 3' region of a cytokine sequence and a

- 36 -

second primer is complementary to a 5' region of a cytokine

sequence. Preferably, the primers are complementary to

distinct, separate regions and are not complementary to each

other. The primers of SEQ ID NOS: 1-10 are merely exemplary

5 of the primers which may be useful in the present invention.

When an amplification method includes the use of

two primers, such as the polymerase chain reaction, the

first primer may be selected from the group consisting of

SEQUENCE ID NOS: 1, 3, 5, 7, and 9, and the second primer

10 may be selected from the group consisting of SEQUENCE ID

NOS: 2, 4, 6, 8, and 10. Any primer pairs which transcribe

nucleic acids toward each other and which are specific for

cytokines may be used in accordance with the methods of the

present invention.

15   Total extraction of RNA is preferably carried out.

As used herein, the term "amplification" refers to

template-dependent processes and vector-mediated propagation

which result in an increase in the concentration of a

specific nucleic acid molecule relative to its initial

20 concentration, or in an increase in the concentration of a

detectable signal. As used herein, the term

template-dependent process is intended to refer to a process

that involves the template-dependent extension of a primer

molecule. The term template dependent process refers to

25 nucleic acid synthesis of an RNA or a DNA molecule wherein

the sequence of the newly synthesized strand of nucleic acid

is dictated by the well-known rules of complementary base

pairing (see, for example, Watson, J. D. et al., In:

- 37 -

Molecular Biology of the Gene, 4th Ed., W. A. Benjamin,
Inc., Menlo Park, Calif. (1987) incorporated herein by
reference in its entirety). Typically, vector mediated
methodologies involve the introduction of the nucleic acid
5   fragment into a DNA or RNA vector, the clonal amplification
of the vector, and the recovery of the amplified nucleic
acid fragment. Examples of such methodologies are provided
by Cohen et al. (U.S. Pat. No. 4,237,224), Maniatis, T. et
al., Molecular Cloning (A Laboratory Manual), Cold Spring
10 Harbor Laboratory, 1982, each incorporated herein by
reference in its entirety.

        A number of template dependent processes are
available to amplify the target sequences of interest
present in a sample. One of the best known amplification
15 methods is the polymerase chain reaction (PCR) which is
described in detail in U.S. Patents 4,683,195, 4,683,202 and
4,800,159, and in Innis et al., PCR Protocols, Academic
Press, Inc., San Diego CA, 1990, each of which is
incorporated herein by reference in its entirety. Briefly,
20 in PCR, two primer sequences are prepared which are
complementary to regions on opposite complementary strands
of the target sequence. An excess of deoxynucleoside
triphosphates are added to a reaction mixture along with a
DNA polymerase (e.g., Taq polymerase). If the target
25 sequence is present in a sample, the primers will bind to
the target and the polymerase will cause the primers to be
extended along the target sequence by adding on nucleotides.
By raising and lowering the temperature of the reaction

- 38 -

mixture, the extended primers will dissociate from the

target to form reaction products, excess primers will bind

to the target and to the reaction products and the process

is repeated. Preferably a reverse transcriptase PCR

5   amplification procedure may be performed in order to

quantify the amount of mRNA amplified. Polymerase chain

reaction methodologies are well known in the art.

        Another method for amplification is the ligase

chain reaction (referred to as LCR), disclosed in EPA No.

10   320,308, incorporated herein by reference in its entirety.

In LCR, two complementary probe pairs are prepared, and in

the presence of the target sequence, each pair will bind to

opposite complementary strands of the target such that they

abut. In the presence of a ligase, the two probe pairs will

15   link to form a single unit. By temperature cycling, as in

PCR, bound ligated units dissociate from the target and then

serve as "target sequences" for ligation of excess probe

pairs. U.S. Patent 4,883,750, incorporated herein by

reference in its entirety, describes an alternative method

20   of amplification similar to LCR for binding probe pairs to a

target sequence.

        Qbeta Replicase, described in PCT Application No.

PCT/US87/00880, incorporated herein by reference in its

entirety, may also be used as still another amplification

25   method in the present invention. In this method, a

replicative sequence of RNA which has a region complementary

to that of a target is added to a sample in the presence of

- 39 -

an RNA polymerase. The polymerase will copy the replicative

sequence which can then be detected.

An isothermal amplification method, in which

restriction endonucleases and ligases are used to achieve

5   the amplification of target molecules that contain

nucleotide 5'-[alpha -thio]triphosphates in one strand of a

restriction site (Walker, G. T., et al., Proc. Natl. Acad,

Sci. (U.S.A.) 1992, 89:392-396, incorporated herein by

reference in its entirety), may also be useful in the

10 amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another

method of carrying out isothermal amplification of nucleic

acids which involves multiple rounds of strand displacement

and synthesis, i.e. nick translation. A similar method,

15 called Repair Chain Reaction (RCR) is another method of

amplification which may be useful in the present invention

and is involves annealing several probes throughout a region

targeted for amplification, followed by a repair reaction in

which only two of the four bases are present. The other two

20 bases can be added as biotinylated derivatives for easy

detection. A similar approach is used in SDA.

Cytokine specific sequences can also be detected

using a cyclic probe reaction (CPR). In CPR, a probe having

a 3' and 5' sequences of non-cytokine specific DNA and

25 middle sequence of cytokine specific RNA is hybridized to

DNA which is present in a sample. Upon hybridization, the

reaction is treated with RNaseH, and the products of the

probe identified as distinctive products generating a signal

which are released after digestion. The original template
is annealed to another cycling probe and the reaction is
repeated. Thus, CPR involves amplifying a signal generated
by hybridization of a probe to a cytokine specific nucleic
5 acid.

Still other amplification methods described in GB
Application No. 2 202 328, and in PCT Application No.
PCT/US89/01025, each of which is incorporated herein by
reference in its entirety, may be used in accordance with
10 the present invention. In the former application,
"modified" primers are used in a PCR like, template and
enzyme dependent synthesis. The primers may be modified by
labelling with a capture moiety (e.g., biotin) and/or a
detector moiety (e.g., enzyme). In the latter application,
15 an excess of labelled probes are added to a sample. In the
presence of the target sequence, the probe binds and is
cleaved catalytically. After cleavage, the target sequence
is released intact to be bound by excess probe. Cleavage of
the labelled probe signals the presence of the target
20 sequence.

Other nucleic acid amplification procedures
include transcription-based amplification systems (TAS)
(Kwoh D., et al., Proc. Natl. Acad. Sci. (U.S.A.) 1989,
86:1173, Gingeras T. R., et al., PCT Application WO
25 88/10315, incorporated herein by reference in their
entirety), including nucleic acid sequence based
amplification (NASBA) and 3SR. In NASBA, the nucleic acids
can be prepared for amplification by standard

phenol/chloroform extraction, heat denaturation of a
clinical sample, treatment with lysis buffer and minispin
columns for isolation of DNA and RNA or guanidinium chloride
extraction of RNA. These amplification techniques involve

5 annealing a primer which has prostate specific sequences.
Following polymerization, DNA/RNA hybrids are digested with
RNase H while double stranded DNA molecules are heat
denatured again. In either case the single stranded DNA is
made fully double stranded by addition of second prostate

10 specific primer, followed by polymerization. The double
stranded DNA molecules are then multiply transcribed by a
polymerase such as T7 or SP6. In an isothermal cyclic
reaction, the RNAs are reverse transcribed into double
stranded DNA, and transcribed once against with a polymerase

15 such as T7 or SP6. The resulting products, whether
truncated or complete, indicate prostate cancer specific
sequences.

        Davey, C., et al., European Patent Application
Publication No. 329,822, incorporated herein by reference in

20 its entirety, disclose a nucleic acid amplification process
involving cyclically synthesizing single-stranded RNA
("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may
be used in accordance with the present invention. The ssRNA
is a first template for a first primer oligonucleotide,

25 which is elongated by reverse transcriptase (RNA-dependent
DNA polymerase). The RNA is then removed from resulting
DNA:RNA duplex by the action of ribonuclease H (RNase H, an
RNase specific for RNA in a duplex with either DNA or RNA).

- 42 -

The resultant ssDNA is a second template for a second

primer, which also includes the sequences of an RNA

polymerase promoter (exemplified by T7 RNA polymerase) 5' to

its homology to its template. This primer is then extended

5   by DNA polymerase (exemplified by the large "Klenow"

fragment of *E. coli* DNA polymerase I), resulting as a

double-stranded DNA ("dsDNA") molecule, having a sequence

identical to that of the original RNA between the primers

and having additionally, at one end, a promoter sequence.

10 This promoter sequence can be used by the appropriate RNA

polymerase to make many RNA copies of the DNA. These copies

can then re-enter the cycle leading to very swift

amplification. With proper choice of enzymes, this

amplification can be done isothermally without addition of

15 enzymes at each cycle. Because of the cyclical nature of

this process, the starting sequence can be chosen to be in

the form of either DNA or RNA.

        Miller, H. I., *et al.*, PCT Application WO

89/06700, incorporated herein by reference in its entirety,

20 disclose a nucleic acid sequence amplification scheme based

on the hybridization of a promoter/primer sequence to a

target single-stranded DNA ("ssDNA") followed by

transcription of many RNA copies of the sequence. This

scheme is not cyclic; i.e. new templates are not produced

25 from the resultant RNA transcripts. Other amplification

methods include "race" disclosed by Frohman, M. A., In: *PCR*

Protocols: A Guide to Methods and Applications 1990,

Academic Press, N.Y.) and "one-sided PCR" (Ohara, O., *et*

- 43 -

al., Proc. Natl. Acad. Sci. (U.S.A.) 1989, *86*:5673-5677),
all references herein incorporated by reference in their
entirety.

Methods based on ligation of two (or more)
5 oligonucleotides in the presence of nucleic acid having the
sequence of the resulting "di-oligonucleotide", thereby
amplifying the di-oligonucleotide (Wu, D. Y. *et al.*,
Genomics 1989, 4:560, incorporated herein by reference in
its entirety), may also be used in the amplification step of
10 the present invention.

Following amplification, the presence or absence
of the amplification product may be detected. The amplified
product may be sequenced by any method known in the art,
including and not limited to the Maxam and Gilbert method,
15 see Sambrook, *supra*. The sequenced amplified product may
then be compared to results obtained with tissue excised
prior to vaccine treatment. Tissue samples obtained prior
to vaccine treatment should be free of cytokine sequences,
particularly IFNγ, TNF, IL2, IL12, and IL13. The nucleic
20 acids may be fragmented into varying sizes of discrete
fragments. For example; DNA fragments may be separated
according to molecular weight by methods such as and not
limited to electrophoresis through an agarose gel matrix.
The gels are then analyzed by Southern hybridization.
25 Briefly, DNA in the gel is transferred to a hybridization
substrate or matrix such as and not limited to a
nitrocellulose sheet and a nylon membrane. A labelled probe
is applied to the matrix under selected hybridization

- 44 -

conditions so as to hybridize with complementary DNA
localized on the matrix. The probe may be of a length
capable of forming a stable duplex. The probe may have a
size range of about 200 to about 10,000 nucleotides in

5   length, preferably about 200 nucleotides in length.
Mismatches such as and not limited to sequences with similar
hydrophobicity and hydrophilicity, will be known to those of
skill in the art once armed with the present disclosure.
Various labels for visualization or detection are known to

10 those of skill in the art, such as and not limited to
fluorescent staining, ethidium bromide staining for example,
avidin/biotin, radioactive labeling such as ^{32}P labeling, and
the like. Preferably, the product, such as the PCR product,
may be run on an agarose gel and visualized using a stain

15 such as ethidium bromide. See Sambrook et al., supra. The
matrix may then be analyzed by autoradiography to locate
particular fragments which hybridize to the probe.

A diagnostic kit for screening for the efficacy of
an autologous, irradiated, hapten conjugated cell

20 composition comprising in one or more containers, a pair of
primers, wherein one of the primers within said pair is
complementary to a cytokine specific sequence, wherein said
primer is selected from the group consisting of SEQ ID NO:
1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5,

25 SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and
SEQ ID NO: 10, and a means for visualizing amplified DNA;
said kit useful for determining the efficacy of said
composition.

- 45 -

The invention is further illustrated by means of
the following actual examples 1-8 and 11 and prophetic
examples 9-10 and 12-14 which are meant to be illustrations
only and are not intended to limit the present invention to
5   these specific embodiments.

Example 1

Sixty-four patients were treated with metastatic
melanoma using a melanoma vaccine, prepared in accordance
with the methods set forth above, preceded by low dose
10 cyclophosphamide (CY) and monitored for immunological
effects and anti-tumor activity. On day 0, the patients
were given cyclophosphamide 300 mg/M^2 i.v. Three days later,
they were injected intradermally with vaccine consisting of
10×10^6 to 25×10^6 autologous, cryopreserved, irradiated
15 (2500 R) tumor cells mixed with BCG; the tumor cells were
obtained by dissociation of metastatic masses enzymatically
(collagenase and DNAse). This treatment sequence was
repeated every 28 days for 8 treatments.

The toxicity of the therapy was limited to a local
20 inflammatory response at the injection site and mild nausea
and vomiting following cyclophosphamide administration.
There were 40 evaluable patients with measurable metastases;
5 had responses - 4 complete and 1 partial. The median
duration of response was 10 months (7-84 + months). One
25 patient continues to be in remission at 11 years.
Regression occurred not only in skin and nodal metastases,
but also in lung and liver metastases. In 6 additional

patients, an anti-tumor response was observed that seemed peculiar to this vaccine therapy, i.e., the regression of metastatic lesions that appeared after the immunotherapy was begun. In 3 patients this "delayed" regression occurred in

5 two or more tumors.

Delayed-type hypersensitivity (DTH) to autologous, mechanically-dissociated melanoma cells was detectable in only 16% of patients before treatment, as compared with in 46%, 56% and 73% of patients on days 49, 161 and 217,

10 respectively. The increases in delayed type hypersensitivity following immunotherapy were statistically significant by a non-independent t-test; day 0 vs. day 49, p < 0.001; day 0 vs. day 161, p < 0.001; day 0 vs. day 217, p = 0.021. Overall, 26/43 patients (61%) exhibited a positive

15 delayed type hypersensitivity response ($\geq$ 5 mm) to autologous melanoma cells at some time point. Patients also developed strong delayed type hypersensitivity to the enzymes used to prepare the tumor cell suspensions: of 24 patients tested for delayed type hypersensitivity with a

20 mixture of collagenase and DNase (each at 1 μg/ml) after two vaccine treatments, 21 (88%) had responses > 5mm. Anti-tumor responses to the vaccine were strongly associated with delayed type hypersensitivity to mechanically-dissociated, autologous melanoma cells, as indicated by three

25 observations: 1) 8/10 patients who exhibited tumor regression had positive delayed type hypersensitivity; 2) in post-surgical adjuvant patients, there was a highly significant correlation between the intensity of delayed

- 47 -

type hypersensitivity to autologous melanoma cells and the time to recurrence of tumor (r = 0.680, p < 0.001); 3) nine patients who developed delayed type hypersensitivity to the autologous melanoma cells in their original vaccine ("old"

5 tumor) developed new metastases ("new" tumor) that did not elicit delayed type hypersensitivity or elicited a much smaller response. The patients were compared to their condition prior to treatment with the vaccine. The patients treated prior to the vaccine study were removed from

10 treatment one to two months prior to starting the vaccine study. Accordingly, the patients were untreated beginning the vaccine study.

       In three cases we were able to excise regressing tumors for histological examination; such tumors were

15 characterized by an intense infiltration of lymphocytes. In contrast, tumors excised from these patients before immunotherapy consisted of homogeneous masses of malignant cells without significant lymphocytic infiltration.

       This study shows that the use of cyclophosphamide

20 allows the development of an immune response to melanoma-associated antigens in cancer-bearing patients.

Example 2

       Patients with metastatic melanoma were sensitized to DNP by topical application to the upper arm with 1%

25 dinitrochlorobenzene (DNCB) or dinitrofluorobenzene (DNFB). Two weeks later they were injected with a vaccine consisting of 10 x 10^6 to 25 x 10^6 autologous, irradiated melanoma cells

- 48 -

conjugated to DNP and mixed with BCG. Cyclophosphamide 300
mg/M² i.v. was given 3 days before DNCB (or DNFB) or vaccine.
Of 4 evaluable patients, 3 have developed a striking
inflammatory response in tumor masses after 2 vaccine

5   treatments (8 weeks). Patient #1 developed erythema and
swelling in the > 50 large (1-3 cm) dermal metastases on her
leg and lower abdomen, followed by ulceration and drainage
of necrotic material, and some are beginning to regress.
Biopsy showed infiltration with CD4+ CD8+ T lymphocytes.

10   Patient #2 developed erythema and swelling in the skin of
her lower abdomen and groin overlying large (8 cm) nodal
masses. These have not yet regressed, but have changed in
consistency from rockhard to fluctuant. Patient #3
exhibited moderate erythema in the skin overlying 3

15   subcutaneous metastases. All 3 patients have developed
delayed type hypersensitivity to both DNCB and to DNP
conjugated autologous lymphocytes.
The patients were compared to their condition prior to
treatment with the vaccine. The patients treated prior to

20   the vaccine study were removed from treatment one to two
months prior to starting the vaccine study. Accordingly,
the patients were untreated beginning the vaccine study.

Example 3

     Fifteen patients (including 3 patients from

25   Example 2) were treated with metastatic melanoma using a
novel form of immunotherapy, i.e., tumor cell vaccine
conjugated to DNP. Patients were sensitized to DNP by

topical application to the upper arm with 5%

dinitrochlorobenzene. Then every 4 weeks they received

cyclophosphamide 300 mg/M² followed 3 days later by injection

of 10×10^6 to 25×10^6 autologous, irradiated melanoma cells

5   conjugated to DNP. Patients received 6-8 treatments. Most

patients (92%) developed delayed-type hypersensitivity (DTH)

to DNP-conjugated autologous lymphocytes or tumor cells

(mean DTH = 17mm). The vaccine induced a striking

inflammatory response in subcutaneous and nodal metastases

10 in 11/15 patients, consisting of erythema, swelling, warmth,

and tenderness around tumor masses, and, in one case,

purulent drainage. Biopsies showed infiltration with

lymphocytes, which, by immunopathological and flow

cytometric analyses, were mainly CD3+, CD4-, CD8+, HLA-DR+ T

15 cells. The melanoma cells in these tissues strongly

expressed ICAM-1, which serves as an adhesion molecule for T

cells. Thus, DNP-vaccine seems to induce a degree of anti-

melanoma immunity not seen with previously tested

immunotherapy. The patients were compared to their

20 condition prior to treatment with the vaccine. The patients

treated prior to the vaccine study were removed from

treatment one to two months prior to starting the vaccine

study. Accordingly, the patients were untreated beginning

the vaccine study.

25 Example 4

This example examined the therapeutic effects of

DNP-vaccine in patients with surgically-resected metastases

and no clinical evidence of metastatic disease. Forty seven

patients were sensitized to the hapten, DNFB

(dinitrofluorobenzene). Then they were treated by

intradermal injection of autologous, irradiated melanoma

5   cells conjugated to DNP. Additional vaccine injections were

administered ever 28 days for a total of eight treatments.

All patients were periodically tested for Delayed Type

Hypersensitivity, DTH, responses to autologous melanoma

cells, DNP-conjugated autologous lymphocytes, and microbial

10 antigens. In vitro studies were performed with

cryopreserved lymphocytes extracted from metastatic tumors

and/or separated from peripheral blood.

The graph of Figure 12 compares the percent of

patients tumor free in the months following surgery treated

15 with DNP vaccine and non-haptenized control vaccine. The

study examined the therapeutic effects of DNP-vaccine in

patients with surgically-resected metastases and no clinical

evidence of metastatic disease. All patients were

sensitized to the hapten, DNFB (dinitrofluorobenzene). Then

20 they were treated by intradermal injection of autologous,

irradiated melanoma cells conjugated to DNP. Additional

vaccine injections were administered over 28 days for a

total of eight treatments. All patients were periodically

tested for Delayed Type Hypersensitivity, DTH, responses to

25 autologous melanoma cells, DNP-conjugated autologous

lymphocytes, and microbial antigens. In vitro studies were

performed with cryopreserved lymphocytes extracted from

metastatic tumors and/or separated from peripheral blood.

- 51 -

DNP-VACCINE THERAPY OF MELANOMA

DAY OF WEEK	M	W	F	M	T	M	W	TH	M	TH	M	W	M
DAY OF STUDY	-21	-19	-17	-14	-13	0	2	3	28	31	49	51	56
CYCLO PHOSPH AMIDE			X			X			X				REPEAT
DNP VACCINE								X		X			CYCLE
DNFB SENS				X	X								FOR
DNFB CHALL						X							A
APPLY SKIN TESTS	X					X					X		TOTAL
READ SKIN TESTS		X					X					X	OF
OBTAIN PBL	X					X					X		8
OBTAIN SERUM	X					X					X		VACCINES
ROUTINE LABS	X										X		

CY (cyclophosphamide) = 300 mg/M^2 i.v. bolus only before first two vaccines

VACCINE = 5 x 10^6 to 20 x 10^6 autologous, irradiated melanoma cells mixed with BCG

DNFB SENS = 1.0 mg in 0.1 ml acetone-corn-oil applied to ventral upper arm

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- 52 -

DNFB CHALL = 200 μg in 0.1 ml acetone-corn-oil applied to

forearm

APPLY SKIN TESTS = autologous melanoma cells, peripheral

blood lymphocytes (PBL), peripheral blood lymphocytes

5   conjugated to DNP (PBL-DNP), purified protein derivative

(PPD) (skin test for tuberculosis), microbial recall

antigens

*Day 0: PBL, PBL-DNP only

READ SKIN TESTS = mean diameter of induration

10 OBTAIN PBL = 100 cc heparinized blood

ROUTINE LABS = complete blood count (CBC), differential

blood count (diff), platelet count (platelets), SMA-12

(panel of routine lab tests, blood urea nitrogen (BUN)

Sensitization to DNP - Patients initially were

15 sensitized to DNP as follows: On day -17, cyclophosphamide,

300 mg/M^2, was administered as a rapid i.v. infusion. Three

days later, on days -14 and -15, patients were sensitized

with DNFB (dinitrofluorobenzene): 1 mg DNFB dissolved in

acetone-corn oil and applied topically in a volume of 0.1 ml

20 within the confines of a 2 cm diameter steel ring. Two

weeks later, patients were tested for reactivity to DNP by

topical application of 200 μg DNFB and intradermal injection

of DNP-conjugated autologous PBL. Cyclophosphamide was

reconstituted in sterile water and the proper dosage was

25 administered by rapid i.v. infusion.

Vaccine Preparation - Tumor masses were processed.

Cells were extracted by enzymatic dissociation with

- 53 -

collagenase and DNAse and by mechanical dissociation, frozen
in a controlled rate freezer, and stored in liquid nitrogen
until needed. On the day that a patient was to be treated,
the cells were thawed, washed, and irradiated to 2500 R.

5　Then they were washed again and suspended in Hanks balanced
salt solution without phenol red.

　　　　Conjugation of melanoma cells with DNP was
performed. This involved a 30 minute incubation of tumor
cells with dinitrofluorobenzene (DNFB) under sterile

10　conditions, followed by washing with sterile saline.

　　　　The vaccine consists of 5-20 x 10^6 live tumor cells
suspended in 0.2 ml Hanks solution. When BCG is added, it
consisted of 0.1 ml of a 1:10 dilution of Tice BCG. Each
vaccine treatment consisted of three injections into

15　contiguous sites on the upper arms or legs, excluding limbs
ipsilateral to a lymph node dissection.

　　　　Study Procedure - On day 0, patients received
cyclophosphamide 300 mg/M^2 as a rapid i.v. infusion. Three
days later, on day +3, they were injected intradermally with

20　autologous melanoma vaccine. Additional vaccine injections
were administered every·four weeks for a total of eight
treatments. Cyclophosphamide was given only prior to the
first two injections. All vaccines were DNP-conjugated and
mixed with Bacillus Calmette-Guerin (BCG). BCG is the Tice

25　strain (substrain of the Pasteur Institute strain) obtained
from Organon Teknika Corporation, Durham, NC. The freeze-
dried material was reconstituted with 1 ml sterile water and
diluted 1:10 in phosphate-buffered saline, pH 7.2; then 0.1

- 54 -

ml was drawn up, mixed with the vaccine and injected. All
vaccines were injected into the same site (upper arm or
leg).

      Immunological evaluation - Skin-testing was
5 performed by the intradermal injection of 0.1 ml of test
material on the forearm, and delayed type hypersensitivity
was assessed at 48 hours by measuring the mean diameter of
induration. Positive reactions were photographed. The
following materials were tested: 1) 1 x 10^6 irradiated
10 autologous melanoma cells; 2) 3 x 10^6 autologous peripheral
blood lymphocytes, both unconjugated and conjugated to DNP;
3) Hanks solution; 4) PPD-intermediate strength; and 5)
microbial recall antigens - Candida, trichophyton, and
mumps. Also, contact sensitivity to DNFB was tested by
15 applying 200 μg to the skin of the forearm and examining the
area for a circle of induration at 48 hours.

      All patients had blood collected for separation
and cryopreservation of lymphocytes and serum each time
skin-testing was performed (see Table 1 for schedule of
20 blood drawing). Periodically, these were tested for: 1)
proliferative and cytotoxic response to autologous melanoma
cells; and 2) proliferative response to DNP-conjugated
autologous lymphocytes.

- 55 -

Duration of Study

1) Patients were treated with eight courses of
vaccine which required about eight months. Treatment was
then stopped. These patients will be monitored until at
5 least five years has elapsed since their initial surgery.

2) Patients who developed regional recurrence or
distant metastases before the completion of eight treatments
were taken off the study and treated as clinically indicated
(chemotherapy or surgery).

10   The control group consisted of 22 patients with
melanoma metastatic to regional lymph nodes. They underwent
surgical resection of their disease, at which time they had
no clinical evidence of metastatic melanoma. Then, they
received treatment with a non-haptenized, autologous
15 melanoma vaccine. First, they were given cyclophosphamide,
300 mg/M^2. Three days later they were injected intradermally
with the vaccine, which consisted of 10 x 10^6 to 25 x 10^6
irradiated, autologous melanoma cells mixed with BCG. The
cyclophosphamide-vaccine treatment was repeated every 28
20 days. A total of eight treatments was given. The patients
were clinically evaluated every two months.

Only 20% of the control patients were cancer-free
at two years. In contrast, patients treated with the DNP-
vaccine of the invention had significantly higher cancer-
25 free survival as set forth above.

The patients who received haptenized vaccine all
had melanoma metastatic to regional lymph nodes, but no
evidence of distant metastases. Patients in this condition

- 56 -

are routinely treated by surgical resection of the diseased

nodes. Surgical resection renders them clinically disease-

free, but they have an 80-85% chance of developing

metastatic melanoma with two years.

5   The patients in the control group were in the same

clinical condition in order to be comparable to the

haptenized vaccine group. Thus, the control group also

consisted of patients with melanoma metastatic to regional

lymph nodes, but no evidence of distant metastases, who had

10 undergone surgical resection of the diseased nodes. When

treatment was initiated with the non-haptenized vaccine, the

control patients were clinically disease-free, but as

previously noted, 80% developed distant metastases.

           Patients with surgically incurable melanoma were

15 not selected as controls because such patients have a cure

rate approaching zero, and an even shorter survival than

patients with resectable lymph node metastases. Moreover,

it is not possible in such patients to measure disease-free

survival, a parameter that was dramatically prolonged by the

20 vaccine of the present invention.

           A statistical analysis of the data was performed

as follows: Kaplan-Meir plots of disease-free survival and

total survival were constructed. The difference between

DNP-vaccine patients and control patients was analyzed by

25 the Mantel log-rank test. These are standard statistical

methods for analyzing such data. The difference was highly

significant with $p < .01$.

- 57 -

Seventeen patients additional were subsequently
treated according to the protocol outlined above (the size
of the control group was not increased for reasons set forth
above). The results maintained statistically significant
5 differences in disease-free survival and total survival.

Example 5

Administration of an autologous, dinitrophenyl
(DNP)-conjugated melanoma vaccine induces T cell
infiltration of metastatic tumors, and prolongs survival of
10 patients who have undergone lymphadenectomy for bulky
regional metastases. These effects appear to be due to
melanoma-specific T cells. Their generation is contingent
upon T cells with specificity for DNP-modified melanoma
cells (DNP-MEL).

15 Clinical Protocol

All patients had metastatic melanoma and were
undergoing immunotherapy with autologous, DNP-conjugated
tumor vaccine, as previously described in Berd, D., et al.,
Cancer Res., 1991, 51, 273 I, incorporated herein by
20 reference in its entirety. Informed consent was obtained
from the patients. Patients were pre-treated with
cyclophosphamide 300 mg/M^2, see Berd et al. (1986) supra, and
three days later were sensitized to DNFB by topical
application of 0.1 ml of a 1 DNFB solution in acetone-corn
25 oil on two consecutive days. Two weeks later patients were
again given cyclophosphamide, followed 3 days later by

- 58 -

injection of DNP-conjugated melanoma vaccine. DNP-vaccine was repeated every 28 days. Cyclophosphamide was given prior to the first two cycles. The vaccine consisted of 10 x 10^6 - 25 x 10^6 cryopreserved, autologous, irradiated (2500

5 R), DNP-conjugated melanoma cells conjugated to DNP mixed with BCG. All tumor preparations contained lymphocytes which were the residua of tumor-infiltrated lymph node tissue. Serum and PBL were collected at the following time points: day 0 (before sensitization), day 14 (2 weeks after

10 DNFB sensitization), day 63 (after 2 vaccines), day 119 (after 4 vaccines), day 175 (after 6 vaccines), and day 231 (after 8 vaccines).

Cellular Reagents

PBL were separated by density gradient

15 centrifugation on Ficoll metrizoate. They were suspended in freezing medium (RPMI-1640 (Mediatech, Washington DC) + 1% human albumin + 10 dimethyl sulfoxide) frozen in a controlled-rate freezer, and stored in liquid nitrogen. HLA typing of PBL was performed by the Thomas Jefferson

20 University Hospital Clinical Laboratory.

Melanoma cells were enzymatically extracted from metastatic masses according to the method of Berd, D., et al., (1986) supra, incorporated herein by reference in its entirety, and cryopreserved. Cell lines were derived from

25 these suspensions and were maintained in RPMI-1640 with 10% fetal calf serum. Melanoma cell lines from the patients used in this study were distinguished by MHC class I

- 59 -

differences determined by flow cytometric analysis with a
panel of monoclonal antibodies obtained from the American
Type Culture Collection: (HB82 = HLA-A2, HB122 = HLA-A3,
HB164 = HLA-A11,24).

5   Hapten Conjugation

        PBL were DNP-modified by a 30 minute incubation
with aqueous DNFB or DNBS, according to the methods of
Miller, S. D. and H. N. Claman, *J. Immunol.*, 1976, *117*, 1519
and Geczy, A. F. and A. Baumgarten, *Immunology*, 1970, *19*,
10 189, (incorporated herein by reference in their entirety)
respectively; the two methods yielded equivalent results.
For specificity controls, cells were modified with TNP by
incubation with TNBS, or with oxazolone, according to the
methods of Fujiwara, H., et al., *J. Immunol.*, 1980, *124*, 863
15 and Boerrigter, G. H. and R. J. Scheper, *J. Invest.*
Dermatol., 1987, *88*, 3, (incorporated herein by reference in
their entirety) respectively. Hapten conjugation was
repeated with melanoma cells.

Delayed-Type Hypersensitivity (DTH)

20   Cryopreserved PBL were thawed, washed, and
resuspended in Hanks balanced salt solution. The cells were
divided into three groups: unmodified, conjugated to DNP,
and conjugated to TNP. After washing, 1 x 10⁶ melanoma cells
or 3 x 10⁶ PBL were suspended in 0.1 ml Hanks solution and
25 injected intradermally on the forearm. DTH was determined

- 60 -

at 48 hours by measuring the mean diameter of induration.
The DTH assay was repeated with melanoma cells.

        All patients developed DTH to DNP-modified
autologous PBL (Figure 1). DTH responses were evident two
5 weeks after topical application of DNFB (day 14), and then
remained stable throughout the period of monthly vaccine
administration. DNP-conjugated autologous melanoma cell
suspensions elicited stronger DTH than DNP-PBL (mean SE: PBL
= 13.3 mm ± 1.3 mm, melanoma cells = 21.9 mm ± 3.6 mm; p <
10 0.01). DTH was specific for DNP-modified "self", since
autologous PBL conjugated to TNP elicited no response in 50
patients tested.

Anti-DNP Antibody

        An ELISA was developed by coating microtiter wells
15 with DNP-conjugated PBL. This method was found to be
preferable to coating plates with DNP-conjugated albumin
because it resulted in lower background readings with serum
of pre-immunized patients. DNP-conjugated PBL (5×10^5 in
0.1 ml) were added to each well of 96 flat bottom plate.
20 The cells were fixed to the plate by drying followed by a 5
minute exposure to 100% methanol. Then, the plates were
washed five times with phosphate buffered saline + 0.05 %
Tween-20. Serial dilutions of test sera were added to the
wells and the plate was incubated in a humidified chamber at
25 37° C for 1 hour. After the incubation, the plate was
washed five times, and then horse radish peroxidase-
conjugated goat anti-human immunoglobulin (Cappel

- 61 -

Laboratories, Malvern, PA) was added at predetermined

optimal dilution. For detecting IgG or IgM antibodies,

peroxidase-conjugated goat anti-human IgG or IgM were used,

respectively. After a 1 hour incubation at 37° C, the plate

5 was washed five times and 0.1 ml of substrate (O-

phenylenediamine, Sigma Chemical Co., St. Louis, MO) was

added to each well followed by 50 μl of 0.12 % hydrogen

peroxide. The plate was read in an ELISA plate reader.

The assay was validated using a murine anti-DNP

10 monoclonal antibody (clone SPE-7; Sigma ImmunoChemicals) and

a peroxidase-conjugated antimouse immunoglobulin antibody as

the second step reagent. Subsequently, the positive control

consisted of a serum sample from a patient who had received

multiple injections of DNP-vaccine. Anti-DNP antibody titer

15 of each serum sample was defined as follows:

(peak OD of sample) X (reciprocal of the dilution having an

OD equal to half the peak OD of positive control)

Butler, J. E., *Methods Enzymol.*, 1981, 73, 482.

Anti-DNP antibody developed in 24 out of 27

20 patients tested (Figure 2). In contrast to DTH, antibody

was not induced by DNFB topical application (day 14). In 19

patients, titers increased above pre-immunization levels

after two intradermal injections of DNP-conjugated melanoma

cells (day 63); in 5 additional patients, significant titers

25 were found only after 4 to 6 vaccines. In all patients, IgG

antibody was detected; anti-DNP IgM was found in only three

patients. Anti-DNP antibody cross reacted with TNP, shown

- 62 -

by binding to TNP-modified cells, but not to the unrelated

hapten, oxazolone.

Development of T cell Lines.

   PBL (1 x 10⁶) were mixed with autologous DNP-

5 conjugated B lymphoblastoid cells (1 x 10⁵) in 24 well flat

bottom plates in lymphocyte culture medium. After 7 days of

culture, IL2 100 U/ml (a gift of Cetus Oncology, Emeryville,

CA) was added. Expanding T cell cultures were maintained in

medium + IL2 and were split as needed to maintain a

10 concentration of about 2 x 10⁶ cells in a 22 mm diameter

well. Every 14 days, the cultures were restimulated by

adding autologous DNP-conjugated B lymphoblastoid cells.

Phenotypes were determined by flow cytometry with a panel of

monoclonal antibodies (Becton-Dickinson, San Jose, CA).

15 Separation of CD8+ and CD4+ T cells was accomplished by

indirect panning in which T cells coated with anti-CD8 or

anti-CD4 monoclonal antibodies were adhered to anti-

immunoglobulin-coated dishes using standard techniques

according to the methods of Wysocki, L. J. and V. L. Sato,

20 *Proc. Natl. Acad. Sci. USA*, 1978, 75, 2844, incorporated

herein by reference in its entirety; the adherent cells were

isolated and expanded with DNP-modified stimulators and IL2.

   Phenotypically homogeneous subpopulations of T

cells were obtained by culturing at limiting dilution in

25 round-bottom microtiter wells in lymphocyte culture medium

containing 2 x 10⁵ irradiated allogeneic feeder cells, 200

U/ml IL2, and phytohemagglutinin. Wells with growing

- 63 -

lymphocyte colonies were screened for ability to proliferate
in response to DNP-modified B lymphoblastoid cells.
Positive wells were expanded in IL2 and restimulated with
autologous DNP-conjugated B lymphoblastoid cells every 14

5   days.

Lymphoproliferative Responses - PBL were tested as responder
cells. They were suspended in lymphocyte culture medium
(RPMI-1640, 10% pooled human AB˙ serum, insulin-transferrin-
selenite media supplement (Sigma Chemical Co.) 2 mM L-

10 glutamine, 1% non-essential amino acids, 25 mM HEPES buffer,
penicillin + streptomycin) and added to 96-well, round
bottom microtiter plates at 1×10^5 cells/well. Stimulator
cells included: 1) autologous or allogeneic PBL, 2)
autologous or allogeneic B lymphoblastoid lines made by

15 transfection with Epstein-Barr virus, 3) autologous cultured
melanoma cells; they were inactivated by irradiation (5000
R). In most experiments, the responder:stimulator ratio was
1:1. The plates were incubated in a CO_2 incubator at 37°C
for 5 days; then the wells were pulsed with ^{125}I-labeled IUDR

20 (ICN Radiochemical, Costa Mesa, CA) for 6 hours, harvested
with an automatic harvesting device, and counted in a gamma
counter. The mean of triplicate wells was calculated.
Cultured T cells were also tested for a lymphoproliferative
response in accordance with the above methods.

25   PBL, obtained and cryopreserved from four patients
at the time of maximum DTH reactivity to DNP-modified
autologous cells, were thawed and tested for in vitro

- 64 -

proliferative responses. PBL from all four patients

proliferated upon stimulation with DNP-modified cells

(Figure 3). The kinetics of the development of the

proliferative response in one of these patients (DM2) is

5   shown in Figure 4. DNFB application alone (day 14) did not

result in detectable numbers of circulating responding

cells. Reactive PBL were detected after two injections of

DNP-vaccine (day 63) and continued to be detected throughout

the 8 months period of vaccine treatment.

10   The proliferative response to DNP-modified cells

was specific, since neither unconjugated PBL nor PBL

modified with TNP evoked responses (Figure 5). Post-vaccine

PBL also proliferated briskly when stimulated with a DNP-

modified melanoma cell line derived from autologous tumor

15   tissue. When stimulated with allogeneic lymphocytes, PBL

exhibited the expected mixed lymphocyte reaction which was

three to five-fold greater than the DNP responses.

              Circulating T lymphocytes from one of these

patients (DM2) were expanded *in vitro* by culture in IL2 and

20   repeated restimulation with autologous DNP-modified B

lymphoblastoid cells. After four weeks of expansion, the T

cells were 70% CD3+, CD8+ and 30% CD3+, CD4+. They

proliferated when stimulated by autologous, DNP-modified B

lymphoblastoid cells or DNP-modified, cultured melanoma

25   cells, but not by unconjugated autologous cells (Figure 6).

These cells were separated by positive panning into CD8-

enriched and CD4-enriched populations that were 98% pure as

determined by flow cytometry analysis. As shown in Figure

7, both CD4-enriched and CD8-enriched T cells exhibited a
proliferative response to DNP-modified autologous B
lymphoblastoid cells. However, only CD8+ T cells responded
to DNP-modified autologous melanoma cells. This result may
5 have been due to the low constitutive expression (< 5%) of
MHC class II by the melanoma cell line.

Expanded T cells were tested for ability to
produce cytokines when stimulated with autologous, DNP-
modified B lymphoblastoid cells. As shown in Figure 8, they
10 produced gamma interferon but not IL4. To determine whether
both CD4+ and CD8+ T cells were involved in the cytokine
response, sublines that were obtained by plating T cells at
limiting dilution were analyzed. Each of these cultures was
homogeneous in respect to expression of CD4 and CD8. Three
15 of these sublines (two CD4+, one CD8+) were tested for
cytokine response to DNP-modified B lymphoblastoid cells.
All three produced gamma interferon, while none made IL4
(Figure 8).

20 Cytokine Production - T cells were added to round bottom
microtiter plates at 1 x 10^5 cells/well. An equal number of
stimulators (DNP-modified autologous B lymphoblastoid cells)
was added, and supernatants were collected after 18 hours
incubati.   Commercially available ELISA kits were used to
25 measure gamma interferon (Endogen, Boston, MA; sensitivity =
5 pg/ml) and IL4 (R&D Systems, Minneapolis, MN; sensitivity
= 3 pg/ml).

- 66 -

To determine the MHC-dependence of the response,
stimulator cells were pre-incubated with monoclonal
antibodies to MHC class I (W6/32) or MHC class II (L243) at
a concentration of 10 µg/ml for one hour before adding
5 responder cells. Non-specific mouse immunoglobulin at the
same concentration was tested as a negative control.

DNP-reactive CD8+ T cells obtained by panning of
the bulk population were able to be maintained in long-term
(> 3 months) culture in IL2-containing medium by repeated
10 stimulation with DNP-modified autologous B lymphoblastoid
cells; they retained the stable phenotype, CD3+, CD8+. Two
lines of evidence confirmed that their response was MHC
class I restricted: 1) Gamma interferon production was
blocked by pre-incubation of stimulator cells with anti-
15 class I framework antibody, but not by anti-class II
antibody (Figure 9), 2) The T cells were able to respond to
allogeneic DNP-modified stimulators that were matched at one
or both HLA-A loci, but not to stimulators that were HLA-A
mismatched. As shown in Figure 10, T cells proliferated
20 upon stimulation with DNP-modified autologous PBL (HLA-A1,
A2, B8+, Bw6) and with DNP-modified allogeneic PBL that
expressed A1 or A2 or both; no response was elicited by DNP-
modified allogeneic stimulators that were A1 and A2-
negative.

25 Cytotoxicity - Melanoma targets were labeled for two hours
with ^{51}Cr (Amersham Corp, Arlington Heights, IL), and 2500
cells were added to round-bottom microtiter wells. Then

- 67 -

effector cells were added to achieve a series of E:T ratios.
After 6 hours incubation at 37° C supernatants were removed
and counted in a gamma counter. Lysis was defined as:
([CPM $_{test}$ - CPM $_{spontaneous}$]/[CPM $_{total}$ - CPM $_{spontaneous}$]) * 100.

5   The cytotoxicity of the CD8+ T cell line was
tested in a ^{51}Cr-release assay with autologous melanoma cells
as targets. To minimize spontaneous ^{51}Cr release, DNP
modification was accomplished with DNBS rather than DNFB. T
cells lysed DNP-modified autologous melanoma cells but not
10 allogeneic (class I-mismatched) melanoma cells (Figures 11a,
11b). There was a direct relationship between
susceptibility to lysis and the degree of DNP modification,
as determined by the concentration of DNBS used. Neither
autologous nor allogeneic targets modified with TNP were
15 lysed.

Example 6
          Clinical data was collected to suggest that an
autologous, DNP-conjugated melanoma vaccine prolongs
20 disease-free survival (DFS) and total survival (TS) in
melanoma patients with bulky but resectable regional lymph
node metastases. Forty-seven patients underwent standard
lymphadenectomy with resection of metastatic masses. Tumor
cells were enzymatically-dissociated from these tissues and
25 cryopreserved. Vaccines consisted of 10 x 10^6 to 20 x 10^6
irradiated (2500 cGy) melanoma cells, conjugated to DNP and
mixed with BCG. They were injected i.d. every 28 days for a
total of 8 treatments. Cyclophosphamide 300 mg/M^2 i.v. was

given 3 days before the first 2 vaccines only. The DFS and TS of these patients were compared with those of 22 melanoma patients with resected nodal metastases treated previously with an unconjugated vaccine, see Example 4. Of 36 patients

5 with stage 3 melanoma (palpable mass in one lymph node region), 22 are disease-free with a median follow-up of 33 months. Kaplan-Meir analysis projects a 3 year DFS and TS of 59% and 71%, respectively. In contrast, the DFS and TS of stage 3 patients treated with unconjugated vaccine was

10 22% and 27% respectively (p = 0.01, log-rank test). Of 11 stage 4 patients (palpable mass in two lymph node regions), 5 are NED (no evidence of disease) with a median follow-up of 41 months. For both stage 3 and 4 patients, the highest rate of relapse was in the first 6 months, a time when anti-

15 melanoma immunity might not have yet been established. This experiment will be followed by an accelerated schedule of immunizations to reduce the rate of early relapses and improve the overall clinical outcome. The patients were compared to their condition prior to treatment with the

20 vaccine. The patients treated prior to the vaccine study were removed from treatment one to two months prior to starting the vaccine study. Accordingly, the patients were untreated beginning the vaccine study.

Example 7

25 Materials and Methods

Human melanoma tissue and cell lines - Tissue was obtained from patients with metastatic melanoma prior to entry into

- 69 -

the vaccine program and at post vaccine time points. The

clinical protocol for the DNP-vaccine administration was

performed in accordance with Berd, D., *et al., Cancer Res.*

1991 *51*:2731-2734. Following surgery, the tumor specimen

5 was transported to the laboratory, tumor tissue was isolated

from surrounding fascia and connective tissue, and pieces of

tumor measuring 2-4 mm^3 were snap frozen in liquid nitrogen.

Melanoma cell lines from the same specimens were derived

from enzyme digests (DNAase and collagenase) of the tumor

10 and propagated as described by Berd, D., *et al. Cancer Res.*

1986 *46*:2572-2577.

Isolation of RNA and Amplification via RT-PCR - Total RNA

was extracted from frozen tissues by grinding in guanidium

isothiocyanate, followed by isolation using CsCl gradient as

15 described by Lattime, E.C., *et al., J. Immunol.* 1988

144:3422-3428. To minimize the loss of tissue RNA, 15 μg of

E. coli ribosomal RNA (Sigma Chemical Corp., St. Louis, MO)

was added to each sample. Isolated RNA was resuspended in

diethylpyrocarbonate treated (DEPC-tx) (Sigma) deionized

20 distilled water. cDNA synthesis was performed using 10 ug

total RNA, Random Primer (Gibco BRL, Gaithersburg, MD), and

RT buffer in DEPC-tx water. This was incubated at 65°C for

10 min and then placed at 4°C. To this, 10 mM DTT (Gibco

BRL), 0.5 mM each dATP, dCTP, dTTP, dTTP, (Gibco BRL),and

25 500 U MMLV-RT (Gibco BRL) was added to achieve a final

reaction volume of 50 μl. Samples were incubated at 37°C

for 1 hour, then heated to 95°C for 5 min.

- 70 -

For amplification by PCR, 5 ul of each cDNA was then added to MicroAmp reaction tubes (Perkin Elmer, Norwalk, CT) containing PCR Reaction buffer, 0.2 mM each dATP, dCTP, dGTP, dTTP, 1.25 U AmpliTaq DNA Polymerase

5 (Perkin Elmer), MgCl$_2$ concentrations determined to be optimal for each primer pair (final concentrations of 1.5-6.0 mM), and 0.5 mM each of the appropriate primer pairs in a final volume of 50 μl. Primer pairs used in this study included β-actin, TNF-α, IL-4, IFNγ, South San Francisco, CA) and

10 IL10 (Clontech, Palo Alto, CA). β-actin served for a standard for comparison of relative mRNA expression between samples, as well as a control for RT and PCR reactions. PCR samples were amplified using a GeneAmp System 9600 thermocycler Perkin Elmer). Each sample was denatured at

15 94°C for 37 sec, annealed at 55°C for 45 sec, and extended at 72°C for 60 sec for 39 cycles, followed by a 10 min. extension at 72°C.

PCR products and size markers (Novagen, Madison, WI) were separated in a 2.0% agarose gel (FMC BioProducts,

20 Rockland, ME). The gel was stained with ethidium bromide, visualized, and photographed under UV illumination. Electrophoresis of PCR products revealed a band corresponding to the predicted fragment size for each set of primers. Nonreverse transcribed RNA was subjected to

25 amplification by PCR as a control for genomic DNA contamination.

Cryopreserved, enzyme-dissociated cell suspensions of melanoma tissues were found not to be suitable for RNA

analysis- These samples usually expressed mRNA for all
cytokines tested, probably a result of activation by the
dissociation process.

<u>Histology and In-Situ RT-PCR</u> - Routine H&E staining of

5 representative specimens was done by the Department of
Pathology. *In situ* RT-PCR was done on paraffin sections
which were permeablized using proteinase K, treated with
reverse transcriptase and the resultant IL10 DNA amplified
using the same primers as noted above using methodology

10 according to Bagasra, O., et al., *J. Immunol. Meth.* 1993
158:131-145.

<u>Cytokine mRNA in Inflamed, Post-vaccine biopsies</u> - While
metastatic melanoma is characterized by a paucity of
lymphocytic infiltration (Elder *et al.*, "The surgical

15 pathology of cutaneous malignant melanoma." In: W.H. Clark,
Jr., *et al.* (Eds.), *Human Malignant Melanoma*, pp. 100, New
York: Grune and Stratton 1979), administration of DNP-
vaccine induces T cells infiltration in metastatic masses
(Berd *et al.*, 1991 *supra.*) Eight (8) subcutaneous

20 metastases (from 4 patients) that had developed inflammation
following vaccine treatment were studied and compared with 3
subcutaneous metastases excised before vaccine and 4 post-
vaccine metastases that failed to develop an inflammatory
response. Post-vaccine, inflamed biopsies contained mRNA

25 for IFNγ (5/8), IL4 (4/8) or both (3/8). In contrast,
neither IFNγ mRNA nor IL4 mRNA was detected in the 7 control
specimens. All but one of these 15 tissues expressed mRNA

for IL10. Figure 14 shows cytokine mRNA expression for a

representative, T cell-infiltrated post-vaccine biopsy along

with the corresponding histology.

Lymph Node Metastases - A group of biopsies of melanoma

5   lymph node metastases was studied as well. Histologically,

these lesions are characterized by an abundance of

lymphocytes (Figure 15B) that are thought to be the residua

of the tumor-infiltrated lymph node lymphocytes (Cardi, et

al., Cancer Res. 1989 49:6562-6565). Of the 10 lymph node

10 biopsies studied, only one expressed mRNA for IFNγ; this

specimen, and one additional specimen, contained mRNA for

IL4. However, all 10 specimens contained mRNA for IL10.

Figure 15 shows cytokine mRNA expression for a

representative lymph node metastasis along with the

15 corresponding histology.

IL10 Production by Melanoma Metastasis and Cell Lines - As

indicated above, IL10 mRNA expression was seen in 24/25

melanoma metastases (Figure 16). Since it was independent

of IFNγ or IL4 mRNA expression and did not correlate with T

20 cell infiltration, melanoma cells, rather than lymphocytes,

may be the source of IL10. Two approaches were used to test

this hypothesis. First, cell lines derived from two of the

metastatic tumors described above were examined. As

illustrated in Figure 17A, both the cell lines and the

25 tissue from which they were derived expressed IL10 mRNA.

Both cell lines produced IL10, as determined by
assay of culture supernatants after a 72 hour incubation
(IL10 concentrations: 760 pg/ml and 10 pg/ml, respectively).
Second, IL10 mRNA expression on a tissue section of a
5 melanoma metastasis was studied using *in situ* RT-PCR. As
seen in Figure 17B, IL10 mRNA is associated in melanoma
cells and not in non-tumor elements.

<u>TNF mRNA is expressed in melanoma metastasis</u> - mRNA for TNF
in human colon carcinoma biopsies using *in situ*
10 hybridization (Naylor, M.S., et al., *Cancer Res.* 1990
50:4436-4440), and that resistance to TNF is associated with
in vivo tumor growth (Lattime, E.C. and Stutman, O.,
J. Immunol. 1989 *143*:4317-4323). TNF mRNA was detected in
6/23 melanoma specimens. There was an association with DNP-
15 vaccine-induced inflammation: 4/7 T cell-infiltrated post-
vaccine biopsies were positive versus 2/16 pre-vaccine or
non-infiltrated post-vaccine specimens.

Example 8
This example discloses dinitrophenyl modified
20 tumor peptides for cancer immunotherapy.
Epstein barr virus (EBV) was added to B
lymphoblastoid cells in culture. The B lymphoblastoid cells
were transformed into a B cell tumor from the patient's own
lymphocytes. Melanoma from a metastasis was cultured in
25 RPMI 1640 + 10% fetal calf serum or 10% pooled human serum.
The non-adhered cells were washed off with RPMI medium.

- 74 -

When the cells were confluent, they were detached with 0.1%

EDTA and passaged into two flasks. This process continued

for about 10 to about 30 passages. To test for gamma

interferon production by T cells, lymphocytes from a

5 patient's blood were obtained. About 1,000,000 lymphocytes

were mixed with DNP modified autologous melanoma cells to

stimulate T cells. Every seven days, 100 U/ml of

interleukin-2 was added. The T cells were expanded by

passage as disclosed above. The T cells were then

10 restimulated by the DNP modified autologous melanoma cells.

An enriched population of T cells resulted which were

responsive to the DNP modified autologous melanoma cells.

Stimulation was determined by the amount of gamma interferon

production by the T cells. Generally the production of

15 gamma interferon at greater than 15 picograms/ml was

considered. These T cells were then used to test the

peptide.

     Small peptides were extracted from 4 types of

cells, all generated from a single patient: 1) B

20 lymphoblastoid cells, 2) B lymphoblastoid cells modified

with dinitrophenyl (DNP), 3) cultured melanoma cells, 4)

cultured melanoma cells modified with DNP. Cells were

suspended in 0.1% trifluoroacetic acid, dounced, sonicated,

and centrifuged at 100,000 xg for 90 minutes. Material in

25 the supernatant of molecular weight >10,000 was removed by a

Centricon 10 filter. The remaining material was separated

on a reversed phase HPLC column. Individual fractions were

collected, dried, resuspended in culture medium, and added

- 75 -

to autologous B lymphoblastoid cells, which bound and

presented the peptides. These peptide-pulsed B cells were

tested for ability to stimulate a T lymphocyte cell line

that was specifically sensitized to autologous DNP-modified

5 melanoma cells.

Initially the 50 HPLC fractions (10 μl of each

sample) were pooled into five groups of ten fractions each.

As shown in Figure 13, only peptides derived from DNP-

modified melanoma cells (DNP-MEL) or DNP-modified B cells

10 (DNP-LY) were stimulatory, and only pool #2 was positive.

Each of the individual fractions of pool #2 were

analyzed by performing the T cell stimulation test with each

fraction in pool 2; activity was found only in fractions #17

and #18, and DNP-MEL peptide stimulated two-fold more gamma

15 interferon production than DNP-LY.

These results indicate that a single HPLC fraction

of low molecular weight peptide preparation contains the

peptide or peptides responsible for stimulation of T cells

sensitized to DNP modified melanoma cells.

20 Example 9

This example will determine peptide stimulation

inhibition by anti-DNP antibody.

The experiment will be identical to that described

for Example 8 with one exception. After adding peptide to

25 the B lymphoblastoid cells, and just before adding the

responding T cells, varying concentrations (1-100 μg/ml) of

anti-DNP antibody will be added to different samples. The

anti-DNP antibody may be obtained from the ATCC, hybridoma
#CRL-1968, or a similar antibody. If the stimulation is
caused by DNP modified peptides, the antibody will inhibit
it. It is expected that fractions 17 and 18 will be

5 inhibited by the antibody.

Example 10

        Example 10 is expected to determine whether the
responding T cells are CD4+ or CD8+.

        The experiment will be identical to that described

10 for Example 8 with one exception. The responding T cells
will be fractionated into subsets before being added to the
peptide-pulsed B lymphoblastoid cells. This is accomplished
by mixing the T cells with magnetic beads coated with either
anti-CD4 or anti-CD8 antibodies (obtained commercially from

15 Immunotech, Inc., Westbrook, Maine). Then the beads, and
the cells that have bound to them are removed with a magnet.
The non-binding cells are washed in tissue culture medium
(RPMI + 10% pooled human serum), counted, and added to the
microtiter wells for measurement of stimulation.

20 Example 11

        Example 11 discloses dinitrophenyl-modified tumor
membranes for cancer immunotherapy.

        Membranes from cultured melanoma cells from one
patient have been prepared according to the method of Heike

25 et al., J. Immunotherapy 1994 15:165-174, the disclosure of
which is incorporated herein by reference in its entirety.

- 77. -

The melanoma cells were conjugated to dinitrophenyl (DNP) according to the methods of Miller and Claman, *J. Immunology* 1976 *117*:1519-1526, the disclosure of which is incorporated by reference, in its entirety. The cells were suspended in

5 5 volumes of 30 mM sodium bicarbonate buffer with 1 mM phenyl methyl sulfonyl fluoride and disrupted with a glass homogenizer. Residual intact cells and nuclei were removed by centrifugation at 1000 g. Then the membranes were pelleted by centrifugation at 100,000 g for 90 minutes. The

10 membranes were resuspended in 8% sucrose and frozen at -80° until needed. Melanoma cells were similarly prepared for conjugation to dinitrophenyl.

These DNP modified melanoma cell membranes were tested for their ability to stimulate autologous T

15 lymphocytes that had been sensitized to DNP modified intact melanoma cells. This was done by incubating about 100,000 T lymphocytes/well with about 10,000-about 100,000 cell equivalents DNP modified membranes/well, and measuring production of gamma interferon production (greater than 15

20 picograms). This process was repeated by incubating the T lymphocytes with DNP modified melanoma cells. The results revealed that intact melanoma cells and the membranes derived from them were equally effective in stimulating T cells.

25 This experiment, which has been repeated several times (using the same patient sample) with similar results, indicates that DNP modified melanoma membranes can

substitute for DNP modified intact melanoma cells in
inducing a T cell response.

Example 12

        Example 12 will determine if addition of

5   autologous monocytes or dendritic cells augments the T cell

response to tumor membranes.

        Autologous monocytes will be isolated as follows.

Peripheral blood lymphocytes will be separated from

peripheral blood by gradient centrifugation according to the

10 methods of Boyum, A., *Scand. J. Clin. Lab. Invest. 21,* 1968

Suppl 97:77-89, the disclosure of which is incorporated

herein by reference in its entirety. They will be suspended

in tissue culture medium (RPMI-1640 + 10% pooled human

serum) and added to plastic microtiter wells for about two

15 hours in order for monocytes to adhere. Then the non-

adherent cells will be washed off with culture medium.

Various concentrations of GM-CSF (granulocyte macrophage

colony stimulating factor, obtained commercially from

Immunex, Seattle, WA) will be added to stimulate growth of

20 the monocytes. After about 2-3 weeks, the monocytes, now

considered macrophages, will be removed from the plastic

with 0.1% EDTA and added in graded numbers (about 100 to

about 10,000/well) to fresh microtiter wells. Graded

numbers of membranes, prepared from DNP modified autologous

25 tumor cells (quantified as cell equivalents), will be added

to the adherent macrophage monolayer. After about 6 to

about 24 hours, DNP specific autologous T cells will be

added and incubated for an additional 24 hours. Then

supernatants will be collected and tested for production of

cytokines such as and not limited to gamma interferon, IL2,

tumor necrosis factor; or for proliferation or stimulation

5   of T cells such as by ^{125}IUDR, ^{3}H thymidine, or with dyes such

as MTT. For example, ^{125}IUDR will be added and the cells will

be collected on an automatic harvesting device to test for T

cell proliferation. Controls will consist of unstimulated T

cells and T cells stimulated with membranes in the absence

10 of macrophages. The ability of autologous dendritic cells

to enhance the response to membranes will be tested in the

same manner. Dendritic cells will be isolated from

peripheral blood mononuclear cells and grown in tissue

culture according to the method of O'Doherty, U., et al., J.

15 Exp. Med. 1993 178:10678-1078, the disclosure of which is

incorporated herein by reference in its entirety.

Example 13

        Example 13 is expected to determine whether

patients who received DNP modified melanoma vaccine manifest

20 delayed type hypersensitivity (DTH) to autologous DNP-

modified melanoma membranes.

        The study subjects will be patients who have

received repeated doses of DNP modified melanoma cell

vaccine. Membranes will be prepared from autologous DNP

25 modified melanoma cells as described above. Graded numbers

of membranes (about 100 to about 10,000 cell equivalents)

will be washed in PBS, resuspended in PBS, and injected

- 80 -

intradermally on the forearm. DTH will be measured about 48

hours later as the diameter of cutaneous induration.

Controls will consist of autologous unconjugated melanoma

cell membranes and membranes prepared from autologous blood

5 lymphocytes.

Example 14

　　　　　Example 14 is expected to determine whether

macrophages or dendritic cells process allogeneic melanoma

10 membranes and present them in an immunogenic manner to T

cells syngeneic to those macrophages.

　　　　　The procedure will be similar to that described

for Example 12 with the exception that the stimulating

membranes will be prepared from allogeneic DNP conjugated

15 melanoma cells. The hypothesis is that macrophages or

dendritic cells from patient A can *in vitro* process

membranes obtained from the melanoma cells of patient B.

This results in stimulation of T cells from patient A. This

experiment may lead to a strategy for allogeneic

20 immunization. DNP modified membranes prepared from a single

allogeneic melanoma cell line, or pool of allogeneic cell

lines, would be processed by a patient's macrophages or

dendritic cells *in vitro*. Those cells would be used for

immunization.

- 81 -

The disclosures of each patent, patent application
and publication cited or described in this document are
hereby incorporated herein by reference, in their entirety.

Various modifications of the invention in addition
5 to those shown and described herein will be apparent to
those skilled in the art from the foregoing description.
Such modifications are also intended to fall within the
scope of the appended claims.

- 82 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT: Berd, David;
           Eisenlohr, Lawrence; and
           Lattime, Edmund

   (ii) TITLE OF INVENTION:
COMPOSITION COMPRISING A TUMOR CELL EXTRACT AND METHOD OF
USING THE COMPOSITION

   (iii) NUMBER OF SEQUENCES: 10
   (iv) CORRESPONDENCE ADDRESS:
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      (F) ZIP: 19103
   (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE
      (B) COMPUTER: IBM PS/2
      (C) OPERATING SYSTEM: PC-DOS
      (D) SOFTWARE: WORDPERFECT 5.1
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      (A) APPLICATION NUMBER: Unknown
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   (vii) PRIOR APPLICATION DATA:
      (A) APPLICATION NUMBER: 08/203,004
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   (viii) ATTORNEY/AGENT INFORMATION:
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      (C) REFERENCE/DOCKET NUMBER: TJU-1582
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(2) INFORMATION FOR SEQ ID NO: 1:
   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 21 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: nucleic acids

   (iii) HYPOTHETICAL: No

   (iv) ANTI-SENSE: No

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
ATGGATGATG ATATCGCCGC G   21

- 83 -

(3) INFORMATION FOR SEQ ID NO: 2:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 33 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: nucleic acids

        (iii) HYPOTHETICAL: No

        (iv) ANTI-SENSE: Yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
CTAGAAGCAT TTGCGGTGGA CGATGGAGGG GCC   33

(4) INFORMATION FOR SEQ ID NO: 3:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 21 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: nucleic acids

        (iii) HYPOTHETICAL: No

        (iv) ANTI-SENSE: No

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
ATGAAATATA CAAGTTATAT C   21

(5) INFORMATION FOR SEQ ID NO: 4:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 33 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: nucleic acids

        (iii) HYPOTHETICAL: No

        (iv) ANTI-SENSE: Yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
TTACTGGGAT GCTCTTCGAC CTTGAAACAG CAT   33

(6) INFORMATION FOR SEQ ID NO: 5:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 21 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: nucleic acids

- 84 -

        (iii) HYPOTHETICAL: No

        (iv) ANTI-SENSE: No

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
ATGGGTCTCA CCTCCCAACT G   21

(7) INFORMATION FOR SEQ ID NO: 6:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 33 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: nucleic acids

        (iii) HYPOTHETICAL: No

        (iv) ANTI-SENSE: Yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
TCAGCTCGAA CACTTTGAAT ATTTCTCTCT CAT   33

(8) INFORMATION FOR SEQ ID NO: 7:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 32 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: nucleic acids

        (iii) HYPOTHETICAL: No

        (iv) ANTI-SENSE: No

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAGCTGAGAA CCAAGACCCA GACATCAAGG CG   32

(9) INFORMATION FOR SEQ ID NO: 8:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 31 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: nucleic acids

        (iii) HYPOTHETICAL: No

        (iv) ANTI-SENSE: Yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
AGCTATCCCA GAGCCCCAGA TCCGATTTTG G   31

(10) INFORMATION FOR SEQ ID NO: 9:
     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 21 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: nucleic acids

     (iii) HYPOTHETICAL: No

     (iv) ANTI-SENSE: No

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
ATGAGCACTG AAAGCATGAT C   21

(11) INFORMATION FOR SEQ ID NO: 10:
     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 23 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: nucleic acids

     (iii) HYPOTHETICAL: No

     (iv) ANTI-SENSE: No

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
TCACAGGGCA ATGATCCCAA AGT   23

- 86 -

What is claimed is:

1.   A composition comprising a therapeutically
effective amount of a hapten modified tumor cell extract,
said composition stimulates T cell lymphocytes and is
5 useful for the treatment of cancer.

2.   The composition of claim 1 wherein said
tumor cell extract is selected from the group consisting of
a hapten modified cancer cell membrane, a low molecular
weight peptide from a hapten modified cancer cell, an
10 antigen presenting cell with a low molecular weight peptide
from a hapten modified cancer cell bound thereto, and an
antigen presenting cell with a hapten modified cancer cell
membrane bound thereto.

3.   The composition of claim 1 wherein said
15 tumor cell extract is a low molecular weight peptide from a
dinitrophenyl modified cancer cell.

4.   The composition of claim 1 wherein said
tumor cell extract is a membrane of a dinitrophenyl
modified cancer cell.

20   5.   A composition of claim 1 wherein said tumor
cell extract is an antigen presenting cell with a low
molecular weight peptide from a hapten modified cancer
cell, or a hapten modified cancer cell membrane, bound
thereto.

25   6.   The composition of claim 1 wherein said
tumor cell extract is selected from the group consisting of
an autologous cell and an allogenic cell.

7.   The composition of claim 1 wherein said
tumor is selected from the group consisting of melanoma,
30 breast, lung, colon, breast, kidney, and prostate.

8.   The composition of claim 1 wherein said
tumor is melanoma.

9.   The composition of claim 2 wherein said
hapten is selected from the group consisting of
5 dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5
sulfonic 1-naphtyl) ethylene diamine.

10.   The composition of claim 9 wherein said
hapten is dinitrophenyl.

11.   The composition of claim 1 further
10 comprising an immunological adjuvant.

12.   The composition of claim 11 wherein said
immunological adjuvant is *Bacillus Calmette-Guerin*.

13.   A composition comprising a therapeutically
effective amount of a dinitrophenyl modified cancer cell
15 membrane, said composition stimulates T cell lymphocytes
and is useful for the treatment of cancer.

14.   A composition comprising a therapeutically
effective amount of a low molecular weight peptide from a
dinitrophenyl modified cancer cell, said composition
20 stimulates T cell lymphocytes and is useful for the
treatment of cancer.

15.   A method for treating cancer comprising
administering to a patient a therapeutically effective
amount of cyclophosphamide; administering a therapeutically
25 effective amount of a hapten modified tumor cell extract
wherein said extract stimulates T cell lymphocytes.

16.   The method of claim 15 wherein said tumor is
selected from melanoma, lung, colon, breast, kidney, and
prostate.

- 88 -

17. The method of claim 15 useful for the treatment of cancer selected from the group consisting of melanoma, lung cancer, colon cancer, breast cancer, kidney cancer, and prostate cancer.

5   18. The method of claim 15 wherein said tumor cell extract is selected from the group consisting of a hapten modified cancer cell membrane and a low molecular weight peptide from a hapten modified cancer cell.

19. The method of claim 18 wherein said hapten 10 is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine.

20. The method of claim 18 wherein said hapten is dinitrophenyl.

15   21. The method of claim 15 wherein said tumor cell extract is a low molecular weight peptide from a dinitrophenyl modified cancer cell.

22. The composition of claim 15 wherein said tumor cell extract is a membrane of a dinitrophenyl 20 modified cancer cell.

23. The method of claim 15 wherein said tumor cell extract is selected from the group consisting of an autologous cell and an allogenic cell.

24. The method of claim 15 wherein said 25 therapeutically effective amount of cyclophosphamide comprises administering a dose of about 300 mg/M^2 of cyclophosphamide prior to administration of said composition.

- 89 -

25. The method of claim 15 wherein said composition is mixed with said immunological adjuvant prior to administration.

26. The method of claim 25 wherein said
5 immunological adjuvant is *Bacillus Calmette-Guerin*.

27. The method of claim 15 further comprising sensitizing the patient with a therapeutically effective amount of 1-fluoro-2,4-dinitrobenzene prior to administering cyclophosphamide.

10   28. A method for the treatment of human cancer comprising administering to a patient with a therapeutically effective amount of cyclophosphamide; administering a therapeutically effective amount of a tumor cell extract which stimulates T cell lymphocytes, said
15 composition mixed with an immunological adjuvant; and administering a therapeutically effective amount of a cytokine selected from the group consisting of interleukin-12, interleukin-2, and interleukin-13.

29. The method of claim 28 wherein said tumor
20 cell extract is selected from the group consisting of a hapten modified cancer cell membrane and a low molecular weight peptide from a hapten modified cancer cell.

30. The method of claim 29 wherein said hapten is selected from the group consisting of dinitrophenyl,
25 trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine.

31. The method of claim 29 wherein said hapten is dinitrophenyl.

- 90 -

32. The method of claim 28 wherein said tumor
cell extract is a low molecular weight peptide from a
dinitrophenyl modified cancer cell.

33. The composition of claim 28 wherein said
5 tumor cell extract is a membrane of a dinitrophenyl
modified cancer cell.

34. The method of claim 28 wherein said tumor
cell extract is selected from the group consisting of an
autologous cell and an allogenic cell.

10   35. The method of claim 28 wherein said
therapeutically effective amount of cyclophosphamide
comprises administering a dose of about 300 mg/M^2 of
cyclophosphamide prior to administration of said
composition.

15   36. The method of claim 28 wherein said
composition is mixed with an immunological adjuvant prior
to administration.

37. The method of claim 28 wherein said
immunological adjuvant is *Bacillus Calmette-Guerin*.

20   38. The method of claim 28 wherein said tumor is
selected from melanoma, lung, colon, breast, kidney, and
prostate.

39. The method of claim 28 useful for the
treatment of cancer selected from the group consisting of
25 melanoma, lung cancer, colon cancer, breast cancer, kidney
cancer, and prostate cancer.

40. The method of claim 28 further comprising
sensitizing the patient with 1-fluoro-2,4-dinitrobenzene
prior to administrating cyclophosphamide.

41. A method for treating cancer comprising
administering to a patient a therapeutically effective
amount of cyclophosphamide; administering a therapeutically
effective amount of a tumor cell extract which stimulates T
5 cell lymphocytes, said composition mixed with an
immunological adjuvant; administering a therapeutically
effective amount of a non-haptenized, irradiated
composition comprising a tumor cell extract.

42. A method of screening for cytokine
10 production by tumors to determine the efficacy of an
autologous, irradiated hapten conjugated cell composition
to alleviate cancer in a patient suspected of having
cancer, said method comprising:
        administering said hapten conjugated composition
15 to said patient;
        obtaining a sample comprising nucleic acids from
a patient tissue sample;
        amplifying nucleic acids specific for a cytokine
or amplifying a signal generated by hybridization of a
20 probe specific to a cytokine specific nucleic acid in said
tissue sample; and
        detecting the presence of the amplified nucleic
acids or the amplified signal, wherein the presence of
amplified nucleic acids or amplified signal from said
25 patient tissue sample indicates cytokine production and
determines efficacy of said hapten conjugated composition.

43. The method of claim 42 wherein said hapten
is selected from the group consisting of dinitrophenyl,
trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl)
30 ethylene diamine.

44. The method of claim 42 wherein said patient
tissue sample is a subcutaneous inflammation.

- 92 -

45.   The method of claim 42 wherein said
amplification step comprises hybridization to a cytokine
specific nucleic acid of at least one oligonucleotide which
is complementary to a cytokine specific sequence.

5   46.   The method of claim 42 wherein the nucleic
acids specific for a cytokine comprise nucleic acids
encoding gamma interferon, tumor necrosis factor,
interleukin 2, interleukin 12, and interleukin 13.

47.   The method of claim 42 wherein said
10 amplification step comprises hybridization to a cytokine
specific nucleic acid with a pair of primers, wherein one
primer within said pair is complementary to cytokine
specific sequence.

48.   The method of claim 42 wherein said
15 amplification step comprises performing a procedure
selected from the group consisting of polymerase chain
reaction, ligase chain reaction, repair chain reaction,
cyclic probe reaction, nucleic acid sequence based
amplification, strand displacement amplification, and Qβ
20 replicase.

49.   The method of claim 42 wherein said
amplification step comprises performing a polymerase chain
reaction, wherein said polymerase chain reaction comprises
a first primer and a second primer, wherein said first
25 primer is selected from the group consisting of SEQUENCE ID
NOS: 1, 3, 5, 7, and 9, and said second primer is selected
from the group consisting of SEQUENCE ID NOS: 2, 4, 6, 8,
and 10.

- 93 -

50. The method of claim 42 wherein said amplification step comprises performing a polymerase chain reaction wherein said polymerase chain reaction comprises a first primer and a second primer, wherein said first primer
5 is SEQ ID NO: 3 and said second primer is SEQ ID NO: 4.

51. The method of claim 42 wherein said amplification step comprises performing a polymerase chain reaction, wherein said polymerase chain reaction comprises a pair of primers, wherein one primer of said pair is
10 complementary to a cytokine specific sequence.

52. The method of claim 42 wherein the primer that is complementary to a cytokine specific sequence is selected from the group consisting of SEQUENCE ID NOS: 1 to 10.

15   53. The method of claim 47 wherein the primer that is complementary to cytokine specific sequence is selected from the group consisting of SEQ ID NOS: 1 to 10.

54. The method of claim 47 wherein the primer that is complementary to a cytokine specific sequence is
20 SEQ ID NO: 3.

55. The method of claim 42 wherein said patient tissue sample is a tissue selected from the group consisting of a tumor, saliva, sputum, mucus, bone marrow, serum, blood, urine, lymph, and a tear.

25   56. A diagnostic kit for screening for the efficacy of an autologous, irradiated, hapten conjugated cell composition comprising in one or more containers, a pair of primers, wherein one of the primers within said pair is complementary to a cytokine specific sequence,
30 wherein said primer is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4,

- 94 -

SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ
ID NO: 9, and SEQ ID NO: 10, and a means for visualizing
amplified DNA; said kit useful for determining the efficacy
of said composition.

5   57.   The diagnostic kit of claim 56 wherein said
means for visualizing amplified DNA is selected from the
group consisting of ethidium bromide stain, ^{32}P, and biotin.

           58.   The method of claim 42 wherein said patient
tissue sample is a melanoma tissue.

10   59.   A method of screening for cytokine
production by a tumor to determine the efficacy of an
autologous, irradiated hapten conjugated cell composition
in a patient suspected of having cancer, said method
comprising:
15   obtaining a sample of RNA from a patient tumor
sample;
           reverse transcribing said RNA into DNA;
           amplifying said DNA with polymerase chain
reaction using a pair of primers which are complementary to
20 separate regions of a cytokine sequence; and
           detecting the presence or absence of amplified
DNA wherein the presence of amplified DNA indicates
cytokine production and determines efficacy of said hapten
conjugated composition.

25   60.   The method of claim 59 wherein the
polymerase chain reaction is *in situ* polymerase chain
reaction.

-95-

AMENDED CLAIMS
[received by the International Bureau on 18 November 1996 (18.11.96);
new claims 61-80 added;
remaining claims unchanged (2 pages)]

61. A composition comprising a hapten modified human tumor cell.

62. A composition of claim 61 wherein said tumor is selected from the group consisting of melanoma, lung, colon, breast, kidney, and prostate.

63. A composition of claim 61 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N' (5 sulfonic 1-naphtyl) ethylene diamine.

64. A composition of claim 61 further comprising an adjuvant.

65. A composition of claim 64 wherein said adjuvant is selected from the group consisting of *Bacillus Calmette-Guerin*, cytokines and QS-21.

66. A composition of claim 61 further comprising a carrier.

67. A composition of claim 66 wherein said carrier is selected from the group consisting of saline and culture medium.

68. A composition comprising a hapten modified human tumor cell wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N' (5 sulfonic 1-naphtyl) ethylene diamine and an adjuvant selected from the group consisting of *Bacillus Calmette-Guerin*, cytokines and QS-21.

69. A composition comprising a hapten modified human melanoma tumor cell.

70. A composition comprising a hapten modified human lung tumor cell.

71. A composition comprising a hapten modified human colon tumor cell.

72. A composition comprising a hapten modified human breast tumor cell.

73. A composition comprising a hapten modified human kidney tumor cell.

74. A composition comprising a hapten modified human prostate tumor cell.

75. A composition of claims 69-74 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N' (5 sulfonic 1-naphtyl) ethylene diamine.

76. A composition of claim 75 further comprising a carrier.

AMENDED SHEET (ARTICLE 19)

77. A composition of claim 76 wherein said carrier is selected from the group consisting of saline and culture medium.

78. A composition of claim 75 further comprising an adjuvant selected from the group consisting of *Bacillus Calmette-Guerin*, cytokines and QS-21.

79. A composition of claim 78 further comprising a carrier.

80. A composition of claim 79 wherein said carrier is selected from the group consisting of saline and culture medium.

STATEMENT UNDER ARTICLE 19

Claims 61-80 are newly added on substitute pages 94/1 and
94/2. Support for the newly added claims may be found in the
specification as filed, see for example, page 16, lines 1-2; page
20, lines 11-28; page 23, lines 1-22; page 28, lines 6 and 8; page
63, line 7; page 73, line 26; page 74, line 28.

The claims are believed to be free of prior art.
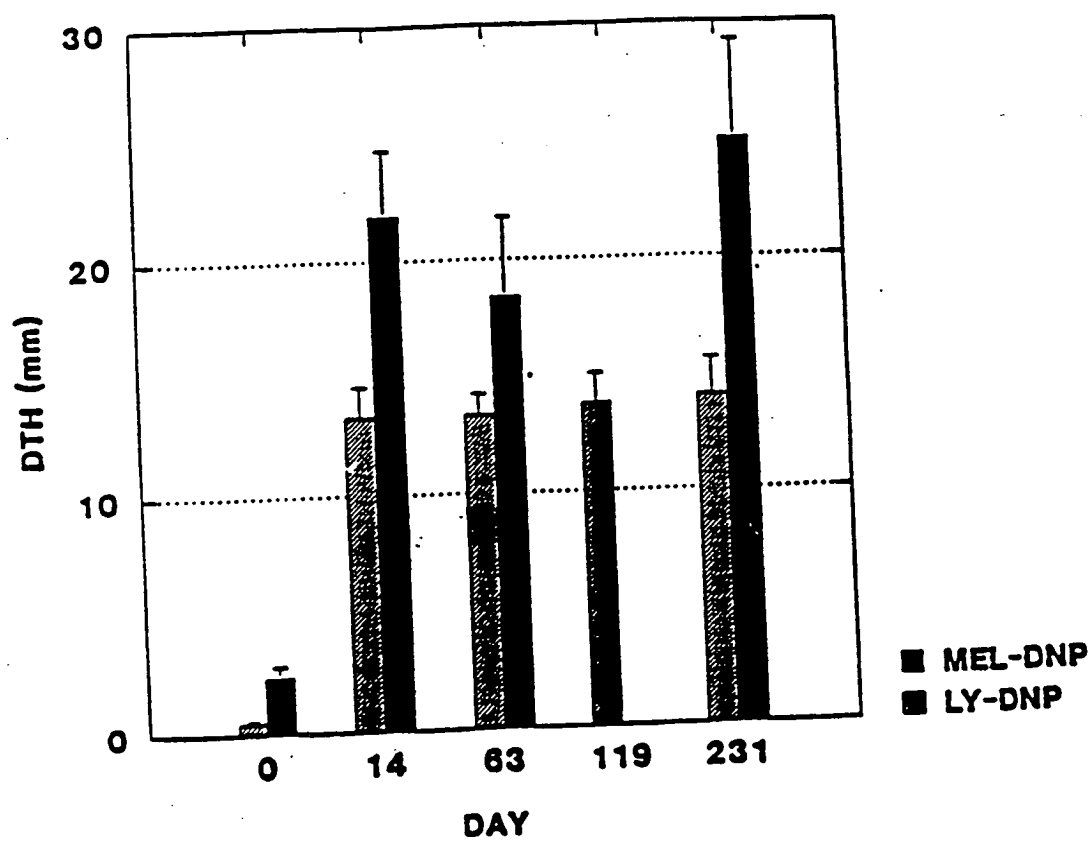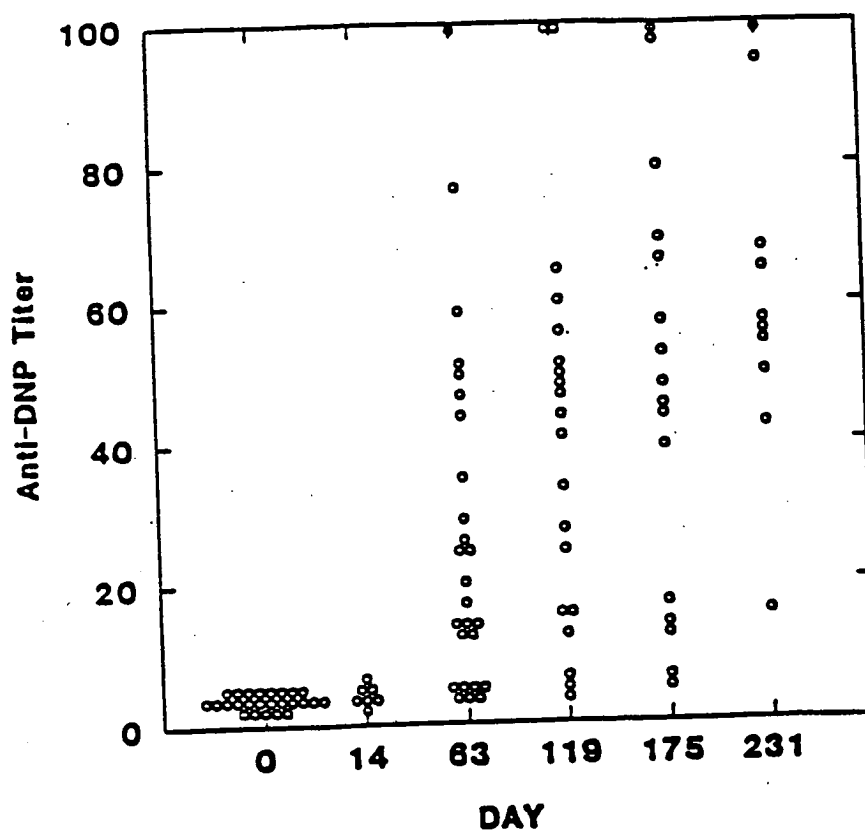
Figure 1

Figure 2

Figure 3

Figure 4
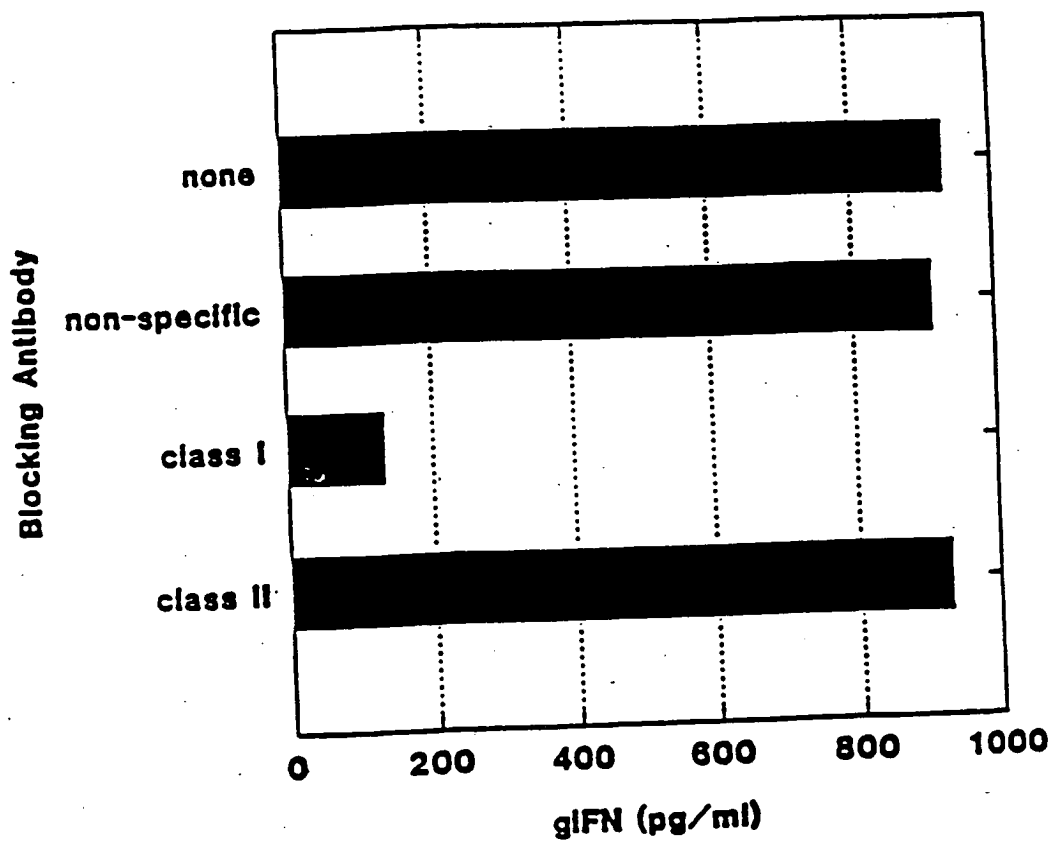
Figure 5

Figure 6
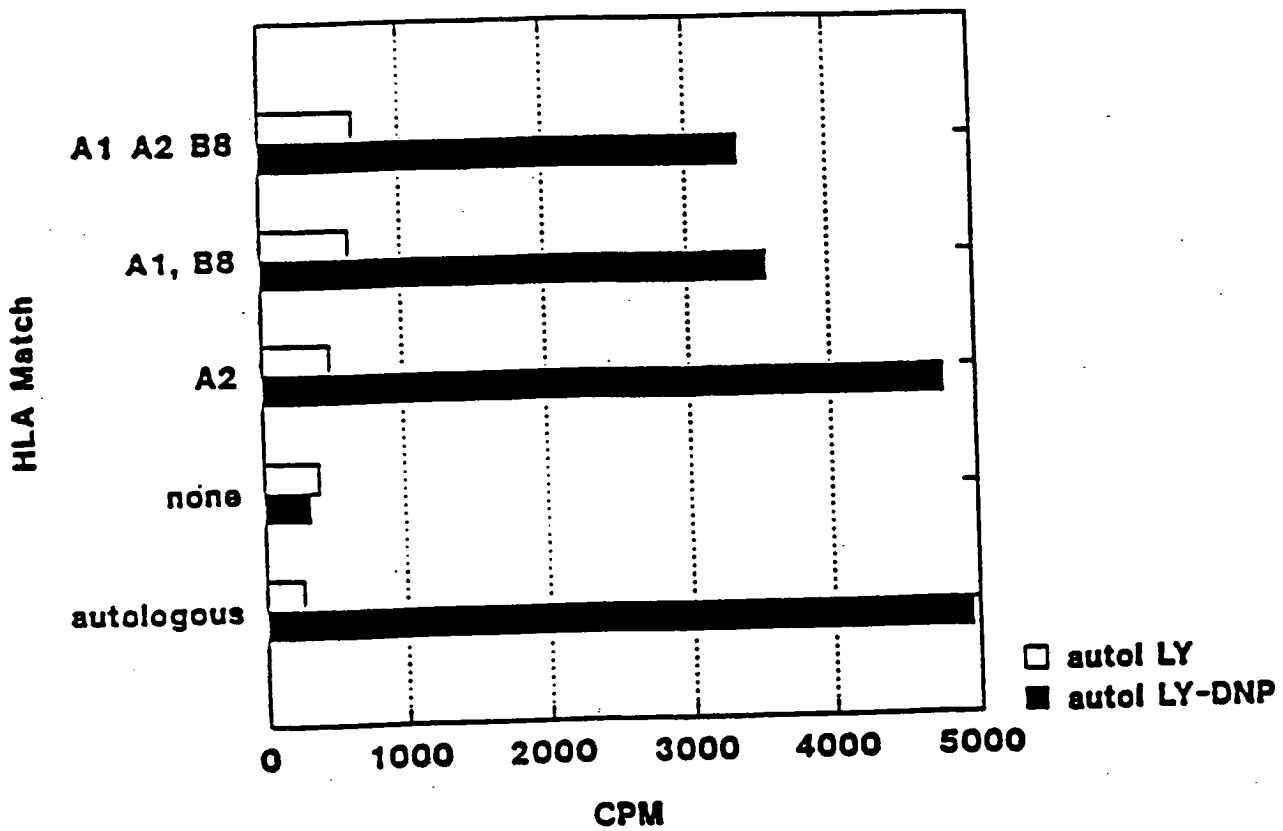
Figure 7

Figure 8
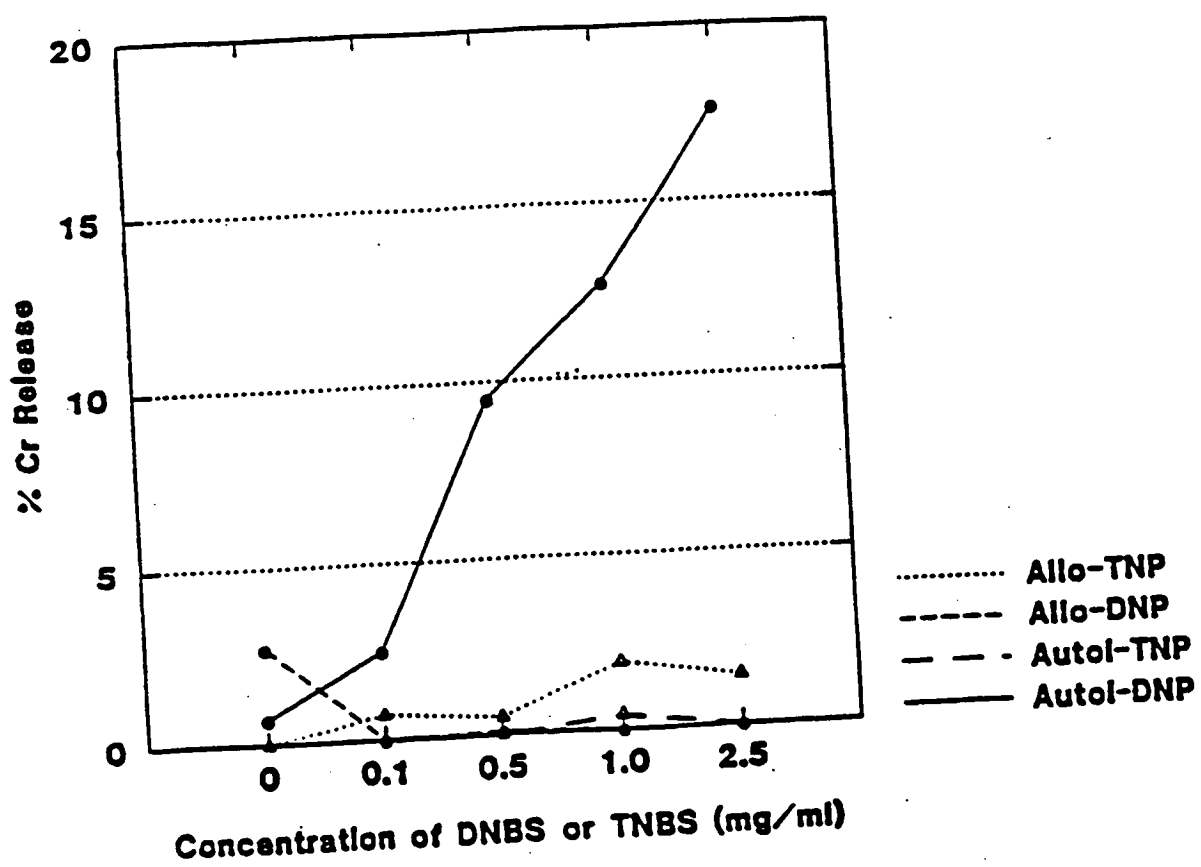
Figure 9

Figure 10
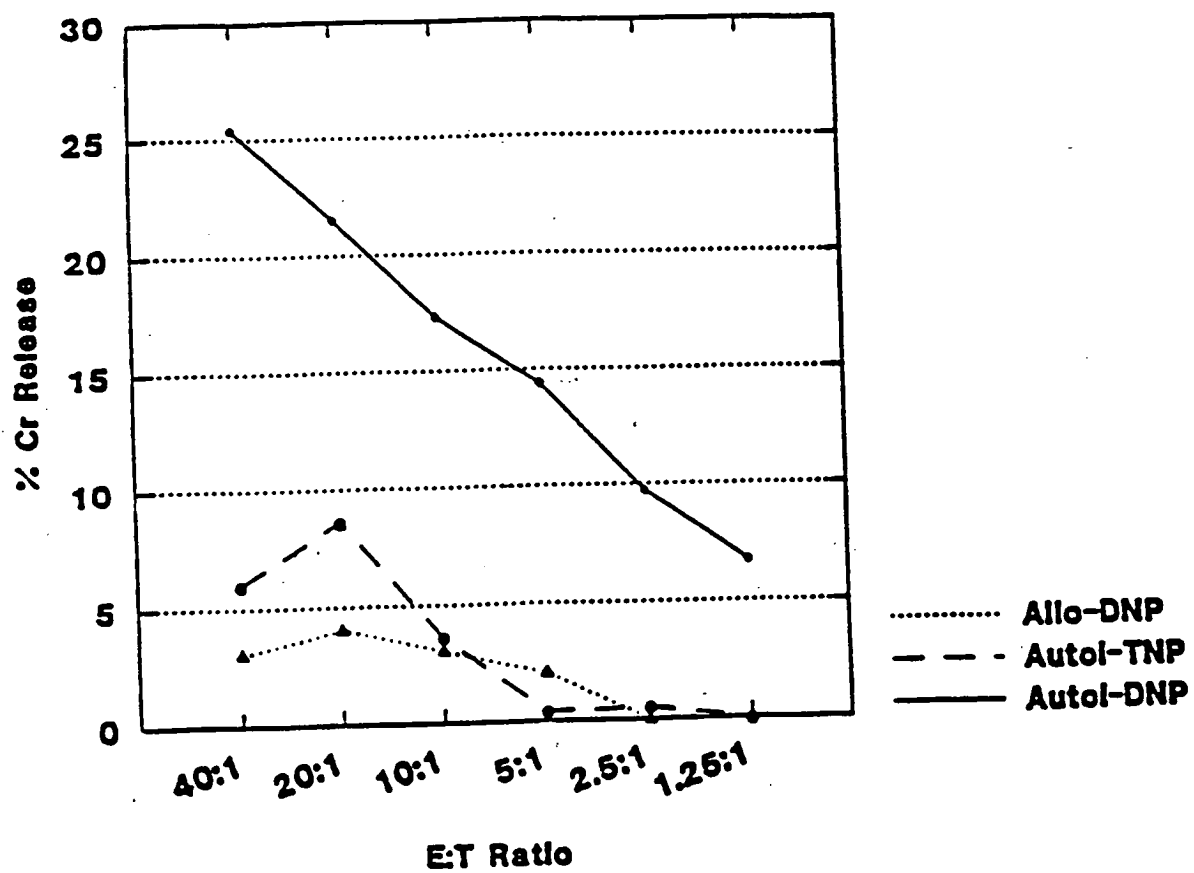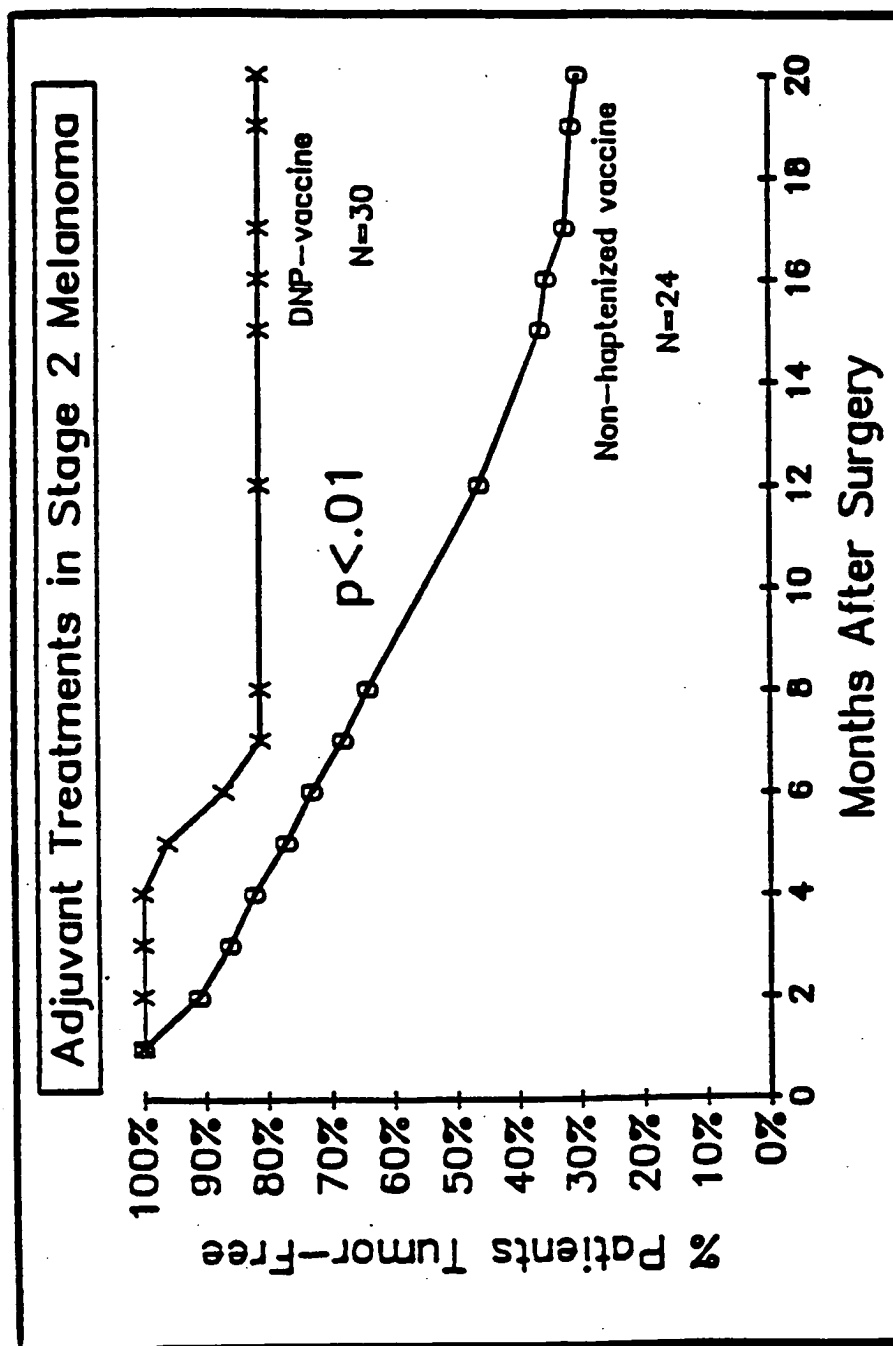
Figure 11A

Figure 11B

FIGURE 12

T Cell Simulation by Peptides from DNP-modified Melanoma Cells
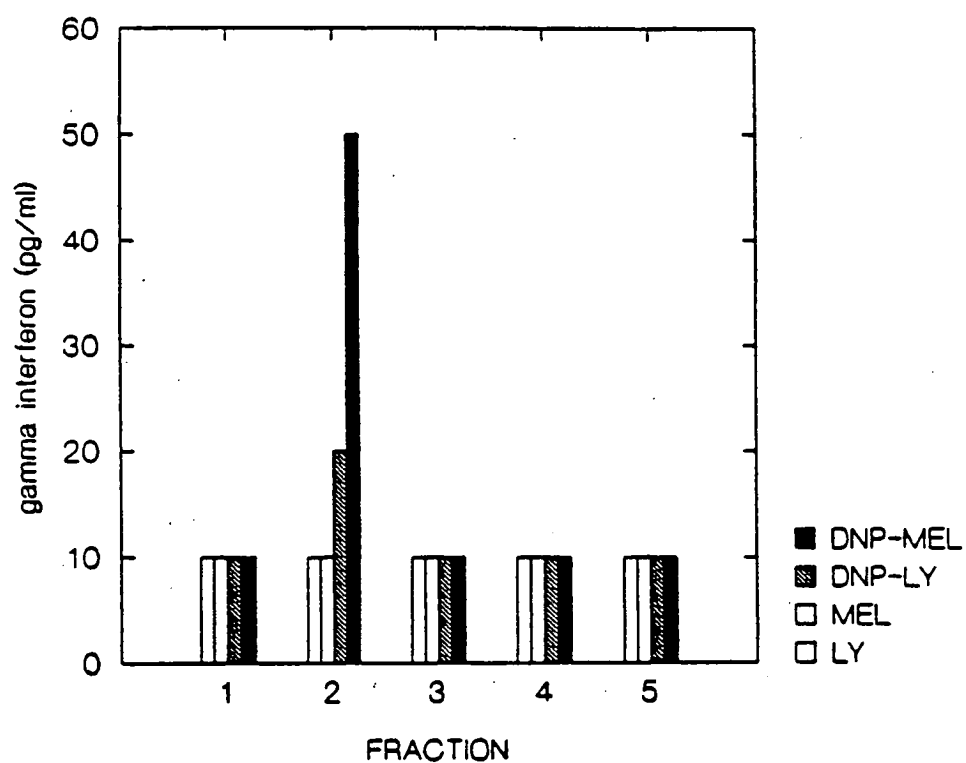
FIGURE 13

A. Inflamed Subcutaneous Melanoma Nodule from DNP-Vaccine Immunized Patient
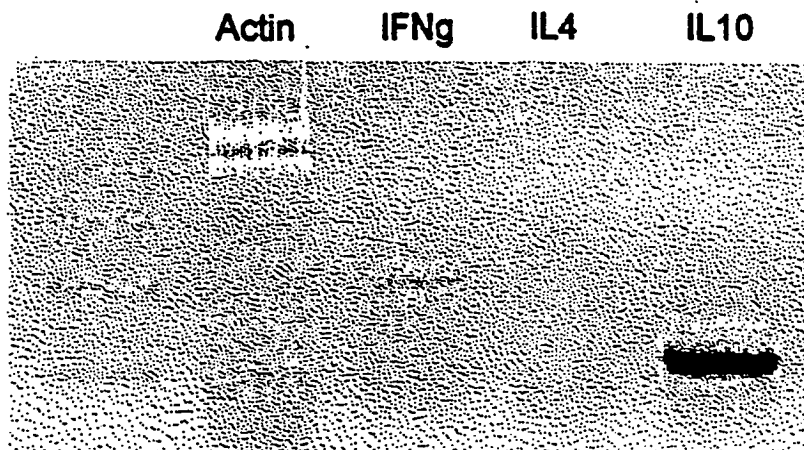
1. mRNA by RT-PCR

2. H&E Stain, Original Magnification 400X
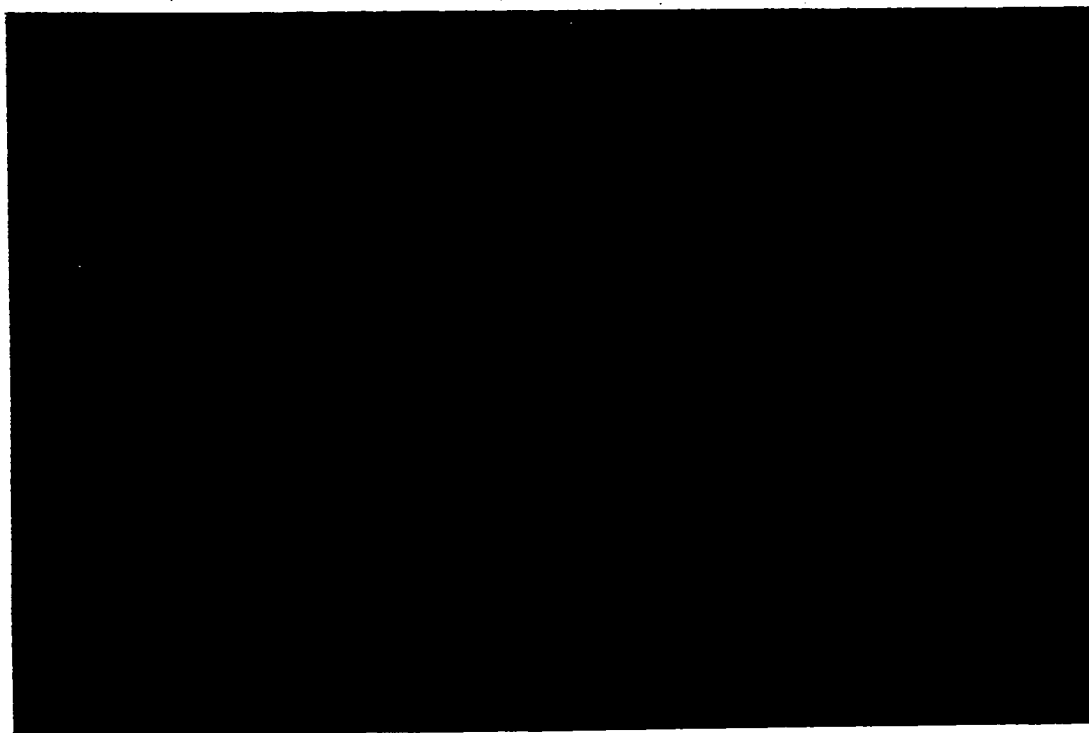
FIGURE 14

B. Lymph Node Metastasis from an Unimmunized Patient

1. mRNA by RT-PCR
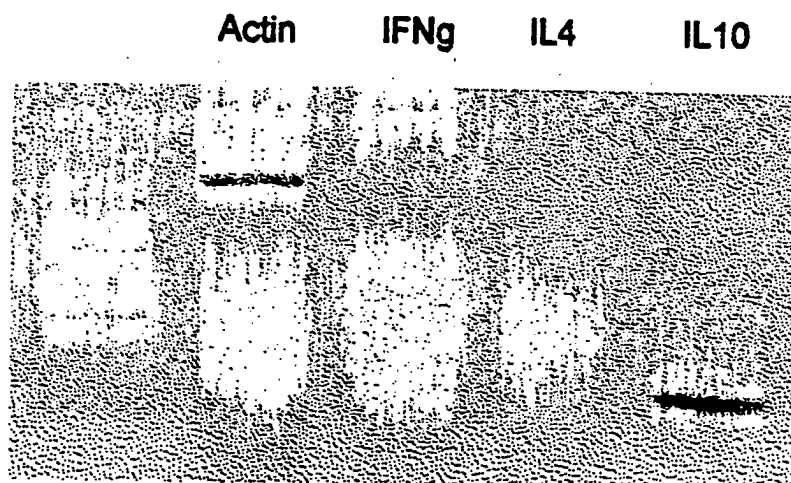
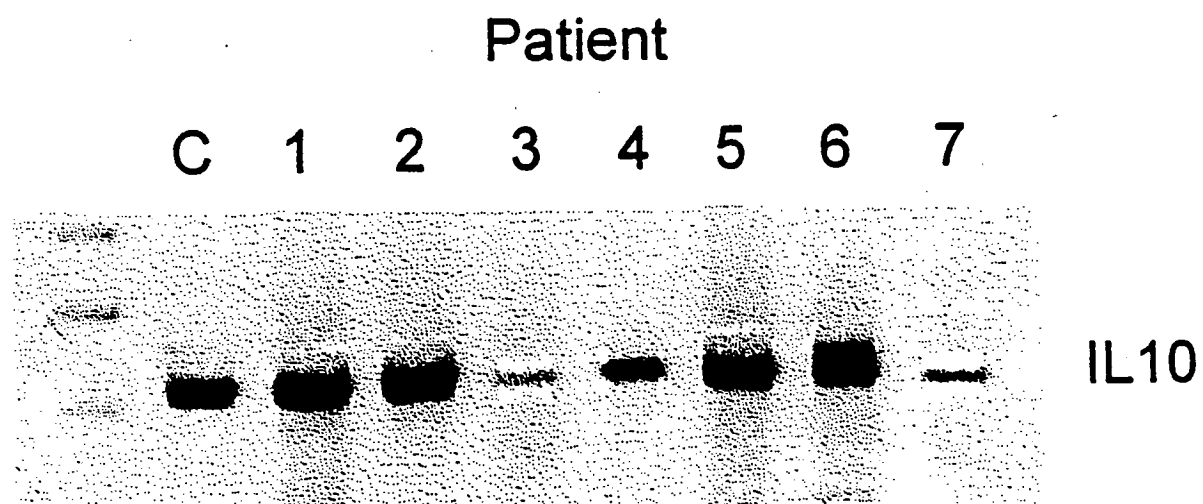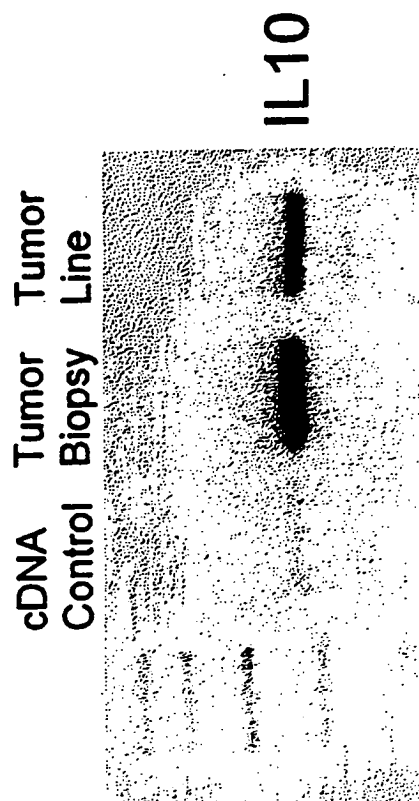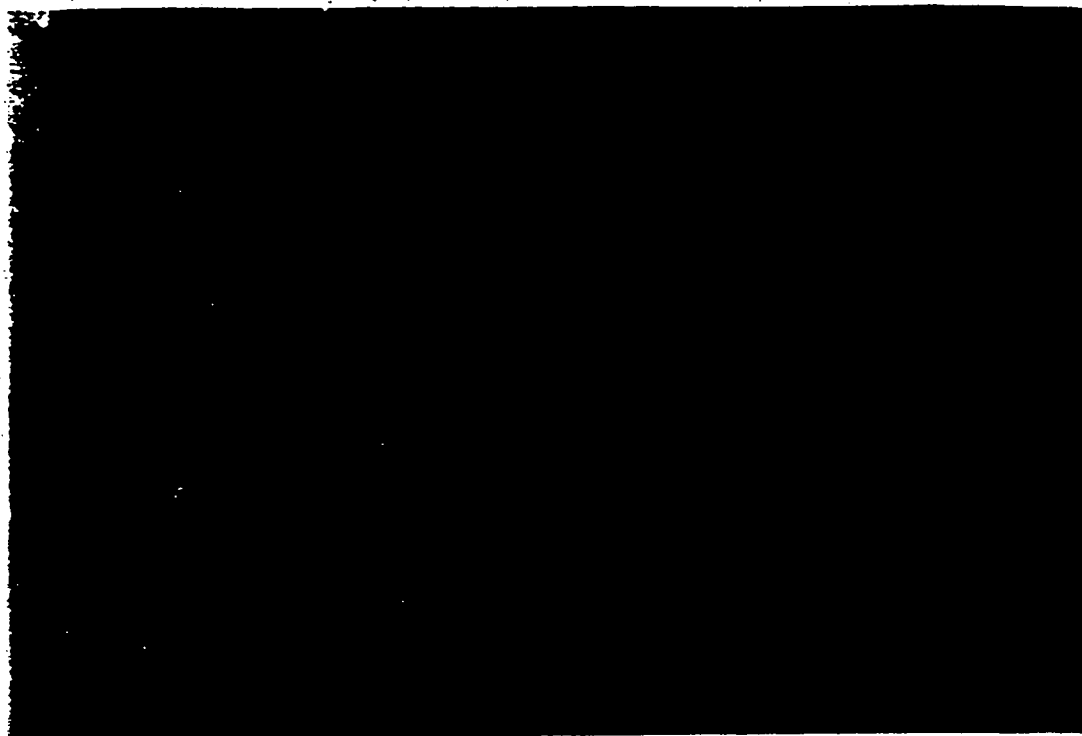## 2. H&E Stain, Original Magnification 400X
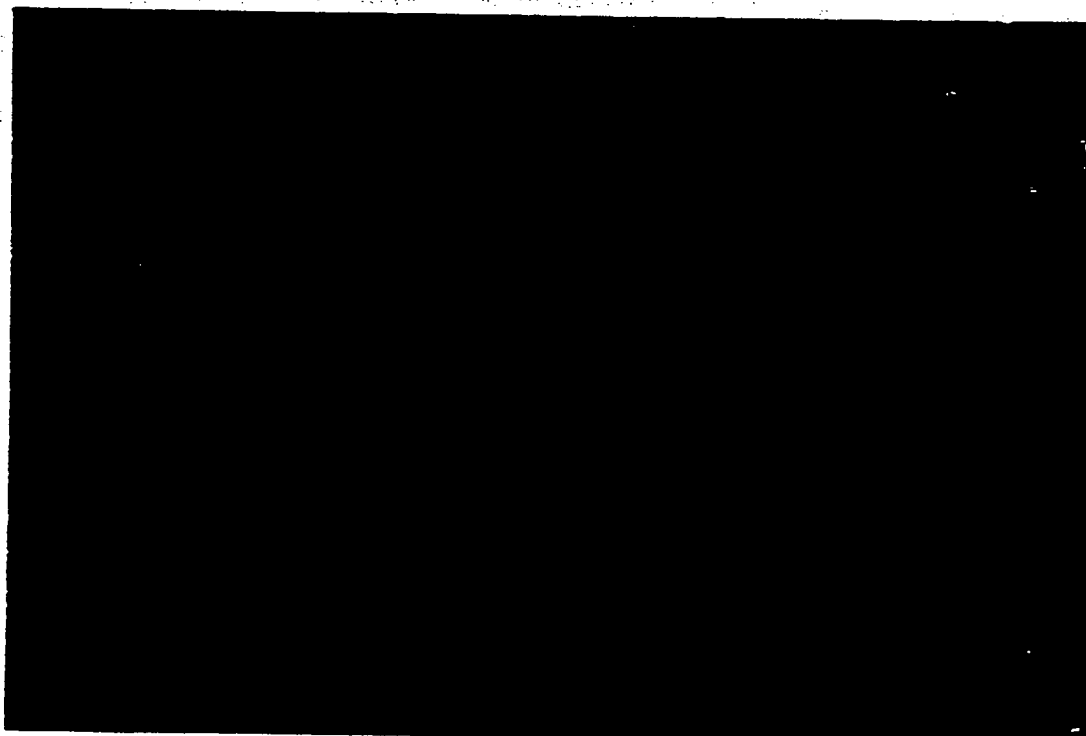
FIGURE 15

FIGURE 16

IL10 mRNA is Expressed by Tumor Biopsy and Biopsy-Derived Cell Line

FIGURE 17

A. Original magnification 100X

B. Original magnification 400X

FIGURE 18

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.2, 85.4, 184.1, 185.1, 193.1, 278.1, 520; 435/6, 91.2, 961; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	SIA et al. Studies in the enhancement of tumour immunity by coupling strong antigens to tumour cells ('heterogenization of tumours'). Helper T cell clones against PPD help other T cells mount anti-tumour responses to PPD-coupled tumour cells. Immunol. April 1984, Vol. 51, pages 755-763, see entire document.	1-12 ---------- 13-40
Y	KOSUGI et al. Cross-reactivity between haptenic muramyl Di- or Tripeptide derivatives and *Mycobacterium bovis* BCG: Potential application for enhancing tumor immunity. Infection & Immunity. December 1986, Vol. 54, No. 3, pages 768-773, see entire document.	1-41

X	Further documents are listed in the continuation of Box C.		☐	See patent family annex.

•	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 SEPTEMBER 1996	**17 SEP 1996**

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer RAY F. EBERT
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BERD et al. Immunization with haptenized, autologous tumor cells induces inflammation of human melanoma metastases. Cancer Research. 15 May 1991, Vol. 51, pages 2731-2734, especially pages 2731-2732.	1-13 and 15-27 ---------- 14 and 28-40
X --- Y	BERD et al. Treatment of human melanoma with a hapten-modified autologous vaccine. Ann. New York Acad. Sci. 16 September 1993, Vol. 690, pages 147-152, see entire document.	1-13 and 15-27 ---------- 14 and 28-40
Y	MACLEAN et al. Active immunization of human ovarian cancer patients against a common carcinoma (Thomsen-Friedenreich) determinant using a synthetic carbohydrate antigen. J. Immunotherapy. April 1992, Vol. 11, pages 292-305, see entire document.	1-41
Y	HELLSTROM et al. Tumor immunology: an overview. Ann. New York Acad. Sci. 16 September 1993, Vol. 690, pages 24-33, especially page 29, paragraph 5.	1-40
Y	WO 90/11764 A1 (FUNG ET AL) 18 October 1990 (18.10.90), Abstract and pages 1-3	41
Y.	WO 87/06840 A1 (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH) 19 November 1987 (19.11.87), see entire document, especially pages 1-4.	41
X --- Y	BYSTRYN, J-C. Tumor vaccines. Cancer & Metastasis Reviews. January 1990, Vol. 9, pages 81-91, especially pages 82-83.	41 ---------- 1-40
Y	HOOVER et al. Prospectively randomized trial of adjuvant active-specific immunotherapy for human colorectal cancer. Cancer. March 1985, Vol. 55, pages 1236-1243, see entire document.	41
Y	KANANGAT et al. Use of quantitative polymerase chain reaction to quantitate cytokine messenger RNA molecules. Molec. Immunol. 1992, Vol. 29, No. 10, pages 1229-1236, see entire document.	42-60
Y	NITTA et al. Expression of tumour necrosis factor-α, -β and interferon-γ genes within human neuroglial tumour cells and brain specimens. Cytokine. March 1994, pages 171-180, see entire document.	42-60

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KAASHOEK, J.G.J. Cytokine production by the bladder carcinoma cell line 5637: Rapid analysis of mRNA expression levels using a cDNA-PCR procedure. Lymphokine & Cytokine Res. 1991, Vol. 10, No. 3, pages 231-235, see entire document.	42-60
Y	PISA et al. Selective expression of interleukin 10, interferon γ, and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies. Proc. Natl. Acad. Sci. USA. August 1992, Vol. 89, pages 7708-7712, see entire document.	42-60
X --- Y	LUSCHER et al. The pattern of cytokine gene expression in freshly excised human metastatic melanoma suggests a state of reversible anergy of tumor-infiltrating lymphocytes. Int. J. Cancer. October 1994, Vol. 57, pages 612-619, see entire document.	42-55 and 58 ---------- 56-57, 59 and 60
Y	CARDING et al. A polymerase chain reaction assay for the detection of cytokine gene expression in small numbers of cells. J. Immunol. Meth. 22 July 1992, Vol. 151, pages 277-287, see entire document.	42-60
X --- Y	GUNNING et al. Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. Mol. Cell. Biol. May 1983, pages 787-795, see entire document.	49, 50, 52-54, 56 ---------- 42-48 and 57-60
Y	WO 95/15334 A1 (CALIFORNIA PACIFIC MEDICAL CENTER RESEARCH INSTITUTE) 08 June 1995 (08.06.95), see entire document, especially cytokine sequences.	49, 52, 53, 56
Y	WO 94/21792 A2 (VIAGENE, INC.) 29 September 1994 (09.29.94) see entire document, especially cytokine sequences.	49, 50, 52-54, 56
Y	GB 2218420 A (BRITISH BIOTECHNOLOGY LIMITED) 15 November 1989 (11.15.89) see entire document, especially IL-4 sequence.	49, 50, 52-54, 56
X --- Y	VIEIRA et al. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. Proc. Natl. Acad. Sci. 15 February 1991, Vol. 88, pages 1172-1176, see entire document.	49, 50, 52-54, 56 ---------- 42-48, 51, 55 and 57-60

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Intu...national application No.

PCT/US96/09511

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 35/12, 38/00, 38/21, 39/00, 39/385, 45/00, 45/05, 47/00
A01N 37/18; C12P 19/34; C12Q 1/68; G01N 33/531

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.2, 85.4, 184.1, 185.1, 193.1, 277.1, 278.1, 520; 435/6, 91.2, 961; 514/2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, WPIDS, BIOSIS, EMBASE
Search terms:
tumor/vaccine/melanoma/hapten/bcg/cyclophosphamide/interleukin/amplif?
cytokine/expression/pcr/Berd,D

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I. Claims 1-40, drawn to compositions and methods for treating cancer using hapten-modified tumor cell
extracts.

Group II. Claim 41, drawn to a method of treating cancer using non-haptenized/irradiated tumor cell extracts mixed
with adjuvant.

Group III. Claims 42-60, drawn to in vitro methods of screening for cytokine production by tumors using nucleic acid
probes and amplification techniques.

The groups listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule
13.2, they lack the same or corresponding special technical features for the following reasons: The special technical
feature of Group I is hapten-modified tumor cell extracts claimed in a product and a method of treating; the special
technical feature of Group II is non-haptenized/irradiated tumor cell extracts claimed in a method of treating; the special
technical feature of Group III is a screening method based on the level of cytokine production, as determined by nucleic
acid amplification and detection. The special technical features are not so linked as to form a single inventive concept
because they differ in compositions (e.g., haptenized vs. non-haptenized cell extracts), method steps (e.g., methods of
treatment vs. methods of screening), objectives (e.g., tumor cell killing vs. tumor cell monitoring), and outcomes (e.g.,
reduction or elimination of tumor vs. detection of tumor or response to treatment). Accordingly, the claims are not so
linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

(51) International Patent Classification 6 :		(11) International Publication Number: **WO 96/40173**
A61K 35/12, 38/00, 38/21, 39/00, 39/385, 45/00, 45/05, 47/00, A01N 37/18, C12P 19/34, C12Q 1/68, G01N 33/531	**A1**	(43) International Publication Date: 19 December 1996 (19.12.96)

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Published
With international search report.
With amended claims and statement.

(54) Title: HAPTEN MODIFIED TUMOR CELL EXTRACT AND METHODS OF TREATING OR SCREENING FOR CANCER

(57) Abstract

A novel composition comprising a hapten modified tumor cell extract prepared from cancer cells, or cancer cell membranes or peptides obtained therefrom is disclosed. Also disclosed are methods of treating cancer comprising administering therapeutically effective amounts of cyclophosphamide and hapten modified tumor cell extracts, with or without adjuvants and cytokines. Also disclosed is a method of screening for cytokine production by tumors in order to determine the efficacy of treatment, wherein cytokin-specific nucleic acids from a patient tissue sample are amplified in order to produce a detectable signal.

"HAPTEN
MODIFIED TUMOR CELL EXTRACT AND METHODS OF TREATING OR SCREENING
FOR CANCER"

REFERENCE TO RELATED APPLICATIONS

        This application is a continuation-in-part of

5 application Serial No. 08/203,004, filed February 28, 1994,

which is a continuation-in-part of application Serial No.

07/985,334, filed December 4, 1992, now U.S. Patent

5,290,551, which is a continuation of application Serial No.

07/520,649, filed May 8, 1990, now abandoned.

10

REFERENCE TO GOVERNMENT GRANTS

        The invention described herein was made in the

course of work under a grant or award from an NIH Cancer

Research grant, grant no. CA39248. The United States

15 Government may have certain rights in this invention. Some

of this invention was disclosed in a Disclosure Document

filed with the U.S. Patent and Trademark Office on April 18,

1990.

- 2 -

BACKGROUND OF THE INVENTION

It was theorized in the 1960's that tumor cells
bear specific antigens (TSA) which are not present on normal
cells and that the immune response to these antigens might
5   enable an individual to reject a tumor. It was later
suggested that the immune response to TSA could be increased
by introducing new immunological determinants on cells.
Mitchison, *Transplant. Proc.*, 1970, 2, 92. Such a "helper
determinant", which can be a hapten, a protein, a viral coat
10   antigen, a transplantation antigen, or a xenogenous cell
antigen, could be introduced into a population of tumor
cells. The cells would then be injected into an individual
who would be expected to be tolerant to the growth of
unmodified tumor cells. Clinically, the hope was that an
15   immunologic reaction would occur against the helper
determinants, as a consequence of which the reaction to the
accompanying TSA is increased, and tumor cells which would
otherwise be tolerated are destroyed. Mitchison, *supra*,
also suggests several modes of action of the helper
20   determinants including 1) that the unmodified cells are
merely attenuated, in the sense that their growth rate is
slowed down or their susceptibility to immunologic attack
increased; 2) that helper determinants merely provide points
of attack and so enable the modified cells to be killed by
25   an immune response not directed against TSA; 3) that the
helper determinants have an adjuvant action such as binding
to an antibody or promoting localization of the cells in the

right part of the body for immunization, in particular, in

lymph nodes.

Fujiwara et al., *J. Immunol.*, **1984a**, *132*, 1571

showed that tumor cells conjugated with the hapten,

5   trinitrophenyl (TNP), could induce systemic immunity against

unmodified tumor cells in a murine system, provided that the

mice were first sensitized to the hapten in the absence of

hapten-specific suppressor T cells. Spleen cells from the

treated mice completely and specifically prevented the

10   growth of tumors in untreated recipient animals. Flood et

al., *J. Immunol.*, **1987**, *138*, 3573 showed that mice immunized

with a TNP-conjugated, ultraviolet light-induced "regressor"

tumor were able to reject a TNP-conjugated "progressor"

tumor that was otherwise non-immunologic. Moreover, these

15   mice were subsequently resistant to challenge with

unconjugated "progressor" tumor. In another experimental

system, Fujiwara et al., *J. Immunol.*, **1984b**, *133*, 510

demonstrated that mice sensitized to trinitrochlorobenzene

(TNCB) after cyclophosphamide pretreatment could be cured of

20   large (10mm) tumors by *in situ* haptenization of tumor cells;

subsequently, these animals were specifically resistant to

challenge with unconjugated tumor cells.

The existence of T cells which cross-react with

unmodified tissues has recently been demonstrated. Weltzien

25   and coworkers have shown that class I MHC-restricted T cell

clones generated from mice immunized with TNP-modified

syngeneic lymphocytes respond to MHC-associated, TNP-

modified "self" peptides. Ortmann, B., et al., *J. Immunol.*,

- 4 -

1992, *148*, 1445. In addition, it has been established that

immunization of mice with TNP-modified lymphocytes results

in the development of splenic T cells that exhibit secondary

proliferative and cytotoxic responses to TNP-modified cells

5   *in vitro*. Shearer, G. M. *Eur. J. Immunol.*, 1974, 4, 527.

The potential of lymphocytes elicited by immunization with

DNP- or TNP-modified autologous cells to respond to

unmodified autologous cells is of considerable interest

because it may be relevant to two clinical problems: I)

10 drug-induced autoimmune disease, and 2) cancer

immunotherapy. In regard to the former, it has been

suggested that ingested drugs act as haptens, which combine

with normal tissue protein forming immunogenic complexes

that are recognized by T cells. Tsutsui, H., et al., *J.*

15 *Immunol.*, 1992, *149*, 706. Subsequently, autoimmune disease,

e.g., systemic lupus erythematosus, can develop and continue

even after withdrawal of absence of the offending drug.

This would imply the eventual generation of T lymphocytes

that cross-react with unmodified tissues.

20   Others have shown that membranes or peptides from

cells, and in one case a peptide from a virus, may elicit a

T cell lymphocyte response *in vitro*. Heike, M., *et al.*, *J*

Immunotherapy, 1994 *15*:165-174, disclose a method of

stimulating mouse and human antitumor cytotoxic T

25 lymphocytes (CTL) with plasma membrane preparations. This

reference identifies differences among the reports of CTL

responses and associates these differences with differences

- 5 -

in immunogenicity of the tumors used and the mode of
immunization, for example.

Others report that the recognition of human
melanoma cells by cytotoxic T cells is mediated by a number
5   of peptides, Slingluff, C., et al., J Immunology 1993
150:2955-2963; Wolfel, T., et al., Int. J. Cancer 1994
57:413-418; and Castelli, C., et al., J. Exp. Med. 1995
181:363-368, the disclosures of each are incorporated herein
by reference in its entirety. Each of these references
10   indicates that there are a number of T cell defined epitopes
which are unique to a given tumor. None of these references
disclose a tumor cell extract which is obtained by hapten
modification of a tumor cell.

The common denominator of these experiments is
15   sensitization with hapten in a milieu in which suppressor
cells are not induced. Spleen cells from cyclophosphamide
pretreated, TNCB-sensitized mice exhibited radioresistant
"amplified helper function" i.e., they specifically
augmented the in vitro generation of anti-TNP cytotoxicity.
20   Moreover, once these amplified helpers had been activated by
in vitro exposure to TNP-conjugated autologous lymphocytes,
they were able to augment cytotoxicity to unrelated antigens
as well, including tumor antigens (Fujiwara et al., 1984b).
Flood et al., (1987), supra, showed that this amplified
25   helper activity was mediated by T cells with the phenotype
Lyt⁻1⁺, Lyt⁻2⁻, L3T4⁻, I⁻J⁺ and suggests that these cells were
contrasuppressor cells, a new class of immunoregulatory T
cell.

- 6 -

Immunotherapy of patients with melanoma has shown
that administration of cyclophosphamide, at high dose (1000
mg/M^2) or low dose (300 mg/M^2), three days before
sensitization with the primary antigen keyhole limpet

5   hemocyanin markedly augments the acquisition of delayed type
hypersensitivity to that antigen (Berd et al., *Cancer Res.*,
1982, *42*, 4862; *Cancer Res.*, 1984a, *44*, 1275). Low dose
cyclophosphamide pretreatment allows patients with
metastatic melanoma to develop delayed type hypersensitivity

10 to autologous melanoma cells in response to injection with
autologous melanoma vaccine (Berd et al., *Cancer Res.*, 1986,
46, 2572). The combination of low dose cyclophosphamide and
vaccine can produce clinically important regression of
metastatic tumor (Berd et al. (1986), *supra*; *Cancer Invest.*,

15 1988a, *6*, 335). Cyclophosphamide administration results in
reduction of peripheral blood lymphocyte non-specific T
suppressor function (Berd et al., *Cancer Res.*, 1984b, *44*,
5439; *Cancer Res.*, 1987, *47*, 3317), possibly by depleting
CD4+, CD45RA+ suppressor inducer T cells (Berd et al.,

20 *Cancer Res.*, 1988b, *48*, 1671). The anti-tumor effects of
this immunotherapy regimen appear to be limited by the
excessively long interval between the initiation of vaccine
administration and the development of delayed type
hypersensitivity to the tumor cells (Berd et al., *Proc.*

25 *Amer. Assoc. Cancer Res.*, 1988c, *29*, 408 (#1626)).
Therefore, there remains a need to increase the therapeutic
efficiency of such a vaccine to make it more immunogenic.

- 7 -

Most tumor immunologists now agree that T
lymphocyte, white cells responsible for tumor immunity,
infiltration into the tumor mass is a prerequisite for tumor
destruction by the immune system. Consequently, a good deal

5 of attention has been focused on what has become known as
"TIL" therapy, as pioneered by Dr. Stephen Rosenberg at NCI.
Dr. Rosenberg and others have extracted from human cancer
metastases the few T lymphocytes that are naturally present
and greatly expanded their numbers by culturing them *in*

10 *vitro* with Interleukin 2 (IL2), a growth factor for T
lymphocytes. Topalian et al., *J. Clin. Oncol.*, 1988, *6*,
839. However this therapy has not been very effective
because the injected T cells are limited in their ability to
"home" to the tumor cite.

15   The ability of high concentrations of IL2 to
induce lymphocytes to become non-specifically cytotoxic
killer cells has been exploited therapeutically in a number
of studies (Lotze et al., *J. Biol. Response*, 1982, *3*, 475;
West et al., *New Engl. J. Med.*, 1987, *316*, 898). However,

20 this approach has been limited by the severe toxicity of
high dose intravenous IL2. Less attention has been given to
the observation that much lower concentrations of IL2 can
act as an immunological adjuvant by inducing the expansion
of antigen activated T cells (Talmadge et al., *Cancer Res.*,

25 1987, *47*, 5725; Meuer et al., *Lancet*, 1989, *1*, 15).
Therefore, there remains a need to understand and attempt to
exploit the use of IL2 as an immunological adjuvant.

- 8 -

Human melanomas are believed to express unique
surface antigens recognizable by T lymphocytes. Old, L. J.,
Cancer Res., 1981, *41*, 361; Van der Bruggen, P., et al.,
Science, 1991, *254*, 1643; Mukherji, B., et al., *J. Immunol.*,
5 1986, *136*, 1888; and Anichini, A., et al., *J. Immunol.*,
1989, *142*, 3692. However, immunotherapeutic approaches to
date have been limited by the difficulty of inducing an
effective T cell-mediated response to such antigens *in vivo*.

There are several models proposed to explicate
10 what appears to be tolerance to human tumor-associated
antigens. They include:

1) Tumor antigen-specific suppressor cells that
down-regulated incipient anti-tumor responses. Mukherji, et
al., *supra*; Berendt, M. J. and R. J. North., *J. Exp. Med.*,
15 1980, *151*, 69.

2) Failure of human tumor cells to elicit T helper
cells or to provide costimulatory signals to those T cells.
Fearon, E. R., et al., *Cell*, 1990, *60*, 397; Townsend, S. E.
and J. P. Allison, *Science*, 1993, *259*, 368; and

20 3) Reduced surface expression of major
histocompatibility products on tumor cells which limits
their recognition by T cells. Ruiter, D. J., *Seminars in
Cancer Biology*, 1991, *2*, 35. None of these hypotheses has
yet been corroborated in a clinical system.

25 The goal of active immunotherapy for tumors is the
development of a productive systemic T cell mediated tumor-
specific immunity. Tumor specific immunity would act both
at the primary tumor site as well as in clearing small

- 9 -

metastatic foci at distant sites. The generation of T cell
immunity has been shown to be a highly regulated response
requiring cell-cell interaction and the production of a
number of cytokines. Of late, studies in a number of human

5 and murine systems have shown that T cell responses can be
separated into two categories termed Type I and II (Mossman,
et al., J. Immunol. 1986 136:2348). Type I responses are
required for the development of delayed type
hypersensitivity (DTH), are associated with macrophage

10 activation and the production of interferon-gamma (IFNγ),
and have been shown to be associated with the resolution of
human leprosy (Yamamura, M., et al., Science 1991 254:277-
279) and murine leishmaniasis (Scott, P., et al.,
Immunological Review 1989 112:161-182). Type II responses

15 are associated with the production of IL4 and IL10,
primarily support antibody responses, and are associated
with the progressive forms of leprosy (Yamamura, M., et al.,
supra) and leishmaniasis (Yamamura et al., supra; and Scott,
P., et al., supra.). In addition to the development of DTH,

20 Type I responses would be expected to enhance the generation
of tumor specific CTL via upregulation of MHC and tumor
associated antigens as well as enhanced antigen presentation
secondary to localized IFNγ production. More recently, Type
I and II responses have been shown to be cross regulating:

25 IFNγ inhibits Type II responses, while IL4 and IL10 inhibit
Type I (Scott, J. Immunol. 1991 147:3149-3155; and
Fiorentino et al., J. Immunol. 1991 146:3444-3451). In the
leishmania system, modulation of cytokines at the lesion

site allows for the conversion of a Type II to a Type I

response, and, consequently, a change from progressive

infection to eradication of the disease (Scott, 1991,

supra).

5   Pisa et al., Proc. Natl. Acad. Sci. USA 1992

89:7708-7712 detected IL10 mRNA in ovarian carcinoma

biopsies, but not in ovarian carcinoma cell lines; they

concluded that the source of IL10 was tumor-infiltrating

lymphocytes. Gastl et al, Int. J. Cancer 1991 55:96-101

10 found that 16/48 tumor cell lines released IL10 into the

culture supernatant; only 3/8 melanoma cell lines were

positive. Finally, Chen et al., Int. J. Cancer, 1994

56:755-760 recently reported that 6/9 cell lines derived

from metastatic melanomas expressed IL10 mRNA. However, the

15 present invention is the first report known to the inventors

of mRNA for IL10 in metastatic melanoma biopsies.

          It is not known whether these observations are

applicable to the human tumor-host relationship, i.e.,

whether the pattern of cytokine production by T cells

20 infiltrating tumors is an indicator of the effectiveness of

the immune response. Patients with metastatic melanoma

treated with an autologous, DNP-modified vaccine develop

inflammatory responses at tumor sites, Berd et al., 1991,

supra. Histologically, these inflamed lesions are

25 characterized by T cell infiltration which is sometimes

associated with tumor cell destruction. The present

invention finds that tumors from DNP-vaccine-treated

patients contain Type I T lymphocytes, which are not

- 11 -

detectable in tumors excised prior to vaccine

administration.

SUMMARY OF THE INVENTION

        The present invention is directed to a treatment

5   for cancer. Compositions and methods of treating cancer are

included in the scope of the present invention. The

compositions of the present invention include a composition

prepared from a tumor cell or tumor cell extract. The

methods of the present invention are directed to treating

10 cancer comprising administering a therapeutically effective

amount of a composition comprising a tumor cell or tumor

cell extract.

BRIEF DESCRIPTION OF THE FIGURES

        Figure 1 displays the kinetics of the development

15 of DTH to DNP-modified autologous PBL and melanoma cells.

Patients with metastatic melanoma treated with DNP-vaccine

were serially skin tested with DNP-modified PBL (LY) or DNP-

modified melanoma cells dissociated from a metastatic mass

(MEL). Each bar indicates the mean DTH response for the

20 group of patients at each time point; error bars = standard

error. For day 119, only responses to PBL were measured.

Sample sizes: days 0, 14, 63 N = 84; day 119, N = 57; day

175, N = 42; day 231, N = 35.

        Figure 2 exhibits antibody response to DNP. Serum

25 obtained at various time points was tested for antibody

(total immunoglobulin) to DNP using an ELISA. The titer was

- 12 -

defined as: (peak OD of sample) X (reciprocal of the
dilution that gave an OD equal to half the peak OD of
positive control).

5   Figure 3 is a graph of proliferative response of
PBL to DNP-modified autologous lymphocytes. PBL were
obtained from four patients receiving DNP-vaccine at the
peak of their DTH responses. The cells were tested for
ability to proliferate to DNP modified autologous PBL (autol
LY-DNP) with unmodified autologous PBL (autol LY) as a
10   control. Cultures were pulsed with ^{125}IUDR on day 6.

Figure 4 shows the kinetics of the proliferative
response to DNP-modified lymphocytes. PBL were serially
collected from patient DM2 while receiving DNP vaccine.
They were cryopreserved and then all samples were tested
15   simultaneously for proliferative response to DNP-modified
autologous PBL (autol LY-DNP). Cultures were pulsed with
^{125}IUDR on day 6.

Figure 5 displays specificity of the proliferative
response to DNP-modified cells. PBL from two patients were
20   tested for proliferative response to autologous PBL, either
unmodified (autol LY), DNP-modified (autol LY-DNP), or TNP-
modified (autol LY-TNP), and to cultured autologous melanoma
cells, either unmodified (MEL) or DNP-modified (MEL-DNP).
Cultures were pulsed with ^{125}IUDR on day 6.

25   Figure 6 is a specificity analysis of expanded T
cells. PBL from patient DM2 were expanded in IL2 and
repeatedly stimulated with autologous DNP-modified B
lymphoblastoid cells. They were tested for proliferative

- 13 -

response to autologous PBL, either unmodifled (autol LY),

DNP modified (autol LY-DNP), or TNP-modified (autol LY-TNP);

cultured autologous melanoma cells, either unmodified (MEL)

or DNP-modified (MELDNP); and allogeneic PBL (Allo LY).

5   Cultures were pulsed with ^{125}IUDR on day 6.

          Figure 7 displays responses of CD8+ and CD4+ T

cells to DNP-modified autologous cells. Expanded T cells

were separated into CD8-enriched or CD4-enriched populations

by positive panning. Then they were tested for

10 proliferative response to autologous PBL, either unmodified

(autol LY), DNP-modified (autol LY-DNP), and to cultured

autologous melanoma cells, either unmodified (MEL) or DNP-

modified (MEL-DNP). Cultures were pulsed with ^{125}IUDR on day

6.

15   Figure 8 shows cytokine production by DNP-reactive

T cells. The DNP-reactive T cell line ("Parent"), and three

subcultures (2F8, 1D7, 1C2), obtained by plating at limiting

dilution, were incubated with autologous DNP-modified B

lymphoblastoid cells for 18 hours; supernatants were

20 collected and assayed for gamma interferon (IFN) and IL4.

          Figure 9 shows blocking of T cell response by

anti-MHC class I monoclonal antibody. Expanded CD8+ T cells

were stimulated with autologous DNP-modified B

lymphoblastoid cells and the cultures were assayed for gamma

25 interferon after I 8 hours. Stimulator cells were pre-

incubated with one of the following: no antibody (none),

non-specific mouse IgG (non-specific), monoclonal antibody

W6/32 (class I), or monoclonal antibody L243 (class II).

- 14 -

Figure 10 exhibits MHC restriction of T cell response. Expanded CD8+ T cells (HLA-AI, A2, B8, Bw6) were tested for ability to proliferate in response to DNP-modified autologous PBL and to DNP modified allogeneic PBL

5   from four other patients. Three of the allogeneic stimulators were matched at one or more class I loci as shown, and the fourth was completely mismatched (A24, A26, B44, B63). Cultures were pulsed with ^{125}IUDR on day 6.

Figure 11 shows graphs of cytotoxicity of DNP-

10   reactive T cells. Melanoma cells, either autologous (autol) or allogeneic class I-mismatched (allo), were used as targets in a 6-hour ^{51}Cr assay. Effector cells were expanded CD8+, DNP-reactive T cells. Figure 11A - target cells were haptenized with various concentrations of DNBS or TNBS. The

15   effector: target cell ratio was 20:1. Figure 11B - Target cells haptenized with 2.5 mg/ml DNBS or TNBS were mixed with effector cells at a series of effector: target (E:T) ratios.

Figure 12 displays a graph of the percentage of patients tumor free in the months following surgery treated

20   with DNP vaccine and non-haptenized control vaccine.

Figure 13 shows the HPLC fractions which were pooled into five groups of ten fractions each. Peptides derived from dinitrophenyl modified melanoma cells (DNP-MEL) or dinitrophenyl modified B cells (DNP-LY) were stimulatory

25   in pool 2.

Figure 14 displays an inflamed subcutaneous melanoma nodule from DNP-vaccine immunized patient expresses mRNA for IFNγ and IL10. Figure 14-1 shows mRNA for

- 15 -

cytokines determined by RT-PCR (lane 1 = size marker; 2 = β-
actin; 3 = IFNγ, 4 = IL4; 5 = IL10); Figure 14-2 is an H&E
stained section of the subcutaneous lesion (400X).

Figure 15 exhibits a lymph node metastasis from an
5 unimmunized patient expresses mRNA for IL10 but not IFNγ.
Figure 15-1 shows mRNA for cytokines determined by RT-PCR
(lane 1 = size marker; 2 = β-actin; 3 = IFNγ; 4 = IL4; 5 =
IL10); Figure 15-2 is an H&E stained section of the lymph
node metastasis (400X).

10 Figure 16 is a gel of IL10 mRNA expressed in human
melanoma metastases. mRNA for cytokines was determined by
RT-PCR (lane 1 = size marker; C = IL10 cDNA control; 1-7 =
patient samples).

Figure 17 is a gel of IL10 mRNA expressed by the
15 melanoma cells. Figure 17 shows IL10 mRNA expression by RT-
PCR from representative tumor biopsy and derived cell line
(lane 1 = size marker; 2 = IL10 cDNA; 3 = tumor biopsy; 4 =
tumor line).

Figure 18 is an *in-situ* RT-PCR from a paraffin
20 section of a non-inflamed melanoma biopsy (A=100x, B=400x).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to cancer
immunotherapy. A novel tumor composition and methods of
treating cancer are included in the scope of the invention.

25 The present invention is directed for use in
treating cancer, including metastatic and primary cancers.
Cancers treatable with the present invention include the

- 16 -

following non-limiting examples: melanoma, breast, lung,
colon, breast, kidney, and prostate. Mammals, particularly
humans, having metastatic cancer may be treated with the
compositions and methods of the present invention.

5   The compositions of the present invention are
prepared from a tumor cell or tumor cell extract. A tumor
cell may be a malignant or pre-malignant cell of any type of
cancer. In accordance with the present invention, pre-
malignant refers to any abnormal cell suggestive of a cancer

10 cell, which is not yet a cancer cell; such as and not
limited to displastic changes in cervical cells which
ultimately lead to cervical cancer, and displastic nevi
which are abnormal skin cells which lead to melanoma. The
tumor cells and extracts preferably originate from the type

15 of cancer which is to be treated. The tumor cells and
extracts may be, and are not limited to, autologous and
allogenic cells dissociated from biopsy specimens or tissue
culture, as well as stem cells and extracts from these
sources. Preferably, the cells and extracts are autologous.

20 Tumor cell extracts of the present invention may be a
peptide isolated from a hapten modified cancer cell or a
cell membrane isolated from a hapten modified cancer cell.

            For purposes of the present invention, peptides
25 are compounds of two or more amino acids and includes
proteins. Peptides will preferably be of low molecular
weight, of about 1,000 kD to about 10,000 kD, more
preferably about 1,000 to about 5,000, which are isolated

from a haptenized tumor cell and which stimulate T cell

lymphocytes to produce gamma interferon. T cells are

lymphocytes which mediate two types of immunologic

functions, effector and regulatory, secrete proteins

5 (lymphokines), and kill other cells (cytotoxicity).

Effector functions include reactivity such as delayed

hypersensitivity, allograft rejection, tumor immunity, and

graft-versus-host reactivity. Lymphokine production and

cytotoxicity are demonstrated by T cell effector functions.

10 Regulatory functions of T cells are represented by their

ability to amplify cell-mediated cytotoxicity by other T

cells and immunoglobulin production by B cells. The

regulatory functions also require production of lymphokines.

T cells produce gamma interferon (IFNγ) in response to an

15 inducing stimulus including and not limited to mitogens,

antigens, or lectins. The peptide may preferably be about 8

to about 20 amino acids, in addition the peptide is

preferably be haptenized. Peptides may be isolated from the

cell surface, cell interior, or any combination of the two

20 locations. The extract may be particular to type of cancer

cell (versus normal cell). The peptide of the present

invention includes and is not limited to a peptide which

binds to the major histocompatibility complex, a cell

surface-associated protein, a protein encoded by cancer

25 oncogenes or mutated anti-oncogenes.

The cancer cell membrane of the present invention

may be all or part of a membrane from a membrane isolated

from a haptenized cancer cell. In accordance with the

- 18 -

definition of cancer cell membrane as set forth for the

present invention, a cancer cell membrane may be isolated

then haptenized, alternatively, a cancer cell may be

haptenized and the membrane subsequently isolated therefrom.

5   The cell extracts are able to stimulate T cells.

Stimulation for purposes of the present invention refers to

proliferation of T cells as well as production of cytokines

by T cells, in response to the cell extract. Membranes and

proteins isolated from hapten modified tumor cells and

10 proteins each independently have the ability to stimulate T

cells. Proliferation of T cells may be observed by uptake

by T cells of modified nucleic acids, such as and not

limited to ^{3}H thymidine, ^{125}IUDR (iododeoxyuridine); and dyes

such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

15 bromide (MTT) which stains live cells. In addition,

production of cytokines such as and not limited to IFNγ,

tumor necrosis factor (TNF), and IL-2. Production of

cytokines is preferably in an amount of greater than 15

picograms/ml, more preferably about 20 to about 30

20 picograms/ml, even more preferably about 50 picograms/ml.

Preferably, the tumor cell extract comprises

cellular materials which are unique, or substantially

specific to, a particular type of cancer. The tumor cells

of the present invention may be live cells. The tumor cells

25 and extracts of the present invention may be irradiated

prior to use. Tumor cells or extracts are irradiated at

about 2500 cGy to prevent the cells from growing after

injection.

- 19 -

The compositions of the invention may be employed
in the method of the invention singly or in combination with
other compounds, including and not limited to other
compositions of the invention. Accordingly, cancer cells
5   and cancer cell extracts may be used alone or co-
administered. For purposes of the present invention, co-
administration includes administration together and
consecutively. In addition, the cancer cell membrane may be
co-administered with the peptide. Further, the cancer cells
10   and/or extracts may be co-administered with other compounds
including and not limited to cytokines such as interleukin-
2, interleukin-4, gamma interferon, interleukin-12, GM-CSF.
The tumor cells and extracts of the invention may also be
used in conjunction with other cancer treatments including
15   and not limited to chemotherapy, radiation, antibodies,
oligonucleotide sequences, and antisense oligonucleotide
sequences.

The compositions of the invention may be
administered in a mixture with a pharmaceutically-acceptable
20   carrier, selected with regard to the intended route of
administration and the standard pharmaceutical practice.
Dosages may be set with regard to weight, and clinical
condition of the patient. The proportional ratio of active
ingredient to carrier naturally depend on the chemical
25   nature, solubility, and stability of the compositions, as
well as the dosage contemplated. Amounts of the tumor cells
and extracts of the invention to be used depend on such
factors as the affinity of the compound for cancerous cells,

- 20 -

the amount of cancerous cells present and the solubility of

the composition. The compounds of the present invention may

be administered by any suitable route, including inoculation

and injection, for example, intradermal, intravenous,

5 intraperitoneal, intramuscular, and subcutaneous.

The compositions of the present invention may be

administered alone or will generally be administered in

admixture with a pharmaceutical carrier selected with regard

to the intended route of administration and standard

10 pharmaceutical practice.

The composition of the present invention is a

therapeutically effective amount of a composition selected

from the group consisting of live tumor cells, tumor cell

extracts, such as a peptide and a cancer cell membrane, and

15 a mixture of tumor cells and one or more tumor cell

extracts. The composition may be mixed with an

immunological adjuvant and/or a pharmaceutically acceptable

carrier. Any known aqueous vehicle useful in drug delivery,

such as and not limited to saline, may be used in accordance

20 with the present invention as a carrier. In addition, any

adjuvant known to skilled artisans may be useful in the

delivery of the present invention. Adjuvants include and

are not limited to Bacillus Calmette-Guerin (BCG);

cytokines, such as and not limited to interleukin-12,

25 interleukin-2; and synthetic adjuvants such as and not

limited to QS-21, (Cambridge Biotech, Worcester, MA)

disclosed by Livingston et al., Vaccine 1994 12:1275, the

disclosure of which is incorporated herein by reference in

its entirety. In the case where the cells and cell extracts
are irradiated and haptenized, the cells may be conjugated
to a hapten and then irradiated. Alternatively, the cells
may be irradiated then conjugated to a hapten. In either
5   case, the extracts are subsequently purified and then may
also be irradiated and/or haptenized. To irradiate and
haptenize the extracts, either method may be performed
first, followed by the other method.

        Alternatively, the tumor cells or tumor cell
10 extracts may be added to antigen presenting cells. The
cancer cell extract may be used to treat cancer together
with another cell type, an antigen presenting cell, selected
from the group consisting of autologous cultured macrophages
and autologous cultured dendritic cells. Macrophages are
15 any large ameboid mononuclear cell, regardless of origin,
such as and not limited to histiocytes and monocytes, which
phagocytose, *i.e.* engulf and destroy, other cells, dead
tissue, degenerated cells, and the like. Macrophages are
antigen presenting cells, which present antigens, including
20 tumor antigens, to cells including T cells. Dendritic cells
are also antigen presenting cells and appear to be closely
related to macrophages, however, dendritic cells are more
efficient antigen presenting cells than macrophages. They
are potent stimulators of T cells and may be isolated from a
25 variety of body organs and tissues including and not limited
to blood, skin (where dendritic cells are referred to as
Langerhans cells), lymphoid tissues.

- 22 -

The antigen presenting cells with peptide or membrane bound thereto, for example, may be used to immunize patients. The patient's blood is obtained and macrophages or dendritic cells are extracted therefrom. High

5 concentrations of the peptide (about 1 ng/ml to about 1 μg/ml, preferably about 10 ng/ml to about 100 ng/ml), or membrane (about 10^5 to about 10^7 cell equivalents, cell equivalents are in relation to the number of starting cells) are incubated with the cells overnight or for about 8 hours.

10 In the case of incubating with membranes, the membranes are phagocytized by the macrophages or dendritic cells. The macrophages or dendritic cells which have phagocytized the membranes are used to immunize the patient, Grabbe, S., et al., Immunology Today 1995 16:117-121, the disclosure of

15 which is incorporated herein by reference in its entirety.

In a preferred embodiment of the invention, the composition comprises a vaccine consisting of about 10 x 10^5 to about 25 x 10^6, more preferably about 5 x 10^6 to about 25 x 10^6, live, irradiated, tumor cells suspended in a

20 pharmaceutically acceptable carrier or diluent, such as and not limited to Hanks solution, saline, phosphate-buffered saline, and water, to which is added an immunological adjuvant, such as and not limited to Bacillus Calmette-Guerin (BCG). The tumor cells and extracts may be

25 conjugated to a hapten. The mixture is injected intradermally into 3 contiguous sites per administration on the upper arms or legs, excluding limbs ipsilateral to a lymph node dissection.

- 23 -

Patients may be immunized to the chemical dinitrophenyl (DNP) by application of dinitrofluorobenzene (DNFB) to the skin. Two weeks later, the patients are injected with a vaccine, which may include irradiated cells

5 haptenized to DNP. The vaccine is reinjected every 4 weeks for a total of eight treatments. The drug cyclophosphamide (CY) may be administered 3 days prior to each vaccine administration to augment the immune response to the tumor cells. A non-haptenized form of the vaccine may be

10 similarly administered.

The vaccine of the present invention may be haptenized or non-haptenized. The haptenized, or chemically-linked, form of the vaccine may include a tumor cell haptenized to dinitrophenyl (DNP) for example. Other

15 haptens include and are not limited to trinitrophenyl (TNP) and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine (AED). A vaccine of tumor cell extracts may similarly be haptenized. In the case of haptenized cancer cell extracts, the extracts, a peptide, and a cancer cell membrane, are

20 isolated from haptenized cancer cells. The present invention also contemplates a non-haptenized vaccine of tumor cells and/or cell extracts.

In the methods of the present invention, a method of treating a patient suspected of having cancer, comprises

25 administering a pharmaceutically acceptable amount of cyclophosphamide, a pharmaceutically acceptable amount of a composition selected from the group consisting of live tumor cells, tumor cell extracts, and a mixture of tumor cells and

- 24 -

tumor cell extracts. Where the composition is a cancer cell

extract, the extract may be a peptide or a membrane isolated

from a haptenized cancer cell. The composition may be mixed

with an immunological adjuvant and/or a pharmaceutically

5   acceptable carrier. The haptenized vaccine may optionally

be followed by administration of a pharmaceutically

acceptable amount of a non-haptenized vaccine. A non-

haptenized vaccine may also be administered in accordance

with the methods of the present invention.

10   The vaccine of the present invention may comprise

tumor cells and/or tumor cell extracts. The tumor cells for

use in the present invention may be prepared as follows.

Tumor masses are processed as described by Berd et al.

(1986), *supra*, incorporated herein by reference in its

15   entirety. The cells are extracted by enzymatic dissociation

with collagenase and DNAse by mechanical dissociation,

frozen in a controlled rate freezer, and stored in liquid

nitrogen until needed. On the day that a patient is to be

skin tested or treated, the cells are thawed, washed, and

20   irradiated to about 2500 R. They are washed again and then

suspended in Hanks balanced salt solution without phenol

red. Conjugation of the prepared cells with DNP is

performed by the method of Miller and Claman, *J. Immunol.*,

1976, *117*, 1519, incorporated herein by reference in its

25   entirety, which involves a 30 minute incubation of tumor

cells with DNFB under sterile conditions, followed by

washing with sterile saline.

- 25 -

Cancer cells of a patient may be conjugated to a
hapten by isolating the membranes and modifying the
membranes or by conjugating the cells to a hapten without
first isolating the membranes.

5   A cancer cell membrane may be prepared by
isolating membranes from non-modified preparation of cancer
cells of a patient. Cells are suspended in about five
volumes of about 30 mM sodium bicarbonate buffer with about
1 mM phenyl methyl sulfonyl fluoride and disrupted with a
10 glass homogenizer. Residual intact cells and nuclei are
removed by centrifugation at about 1000 g. The membranes
are pelleted by centrifugation at 100,000 g for 90 minutes.
The membranes are resuspended in about 8% sucrose and frozen
at about -80°C until needed. To a suspension of membranes
15 (about 5,000,000 cell equivalents/ml), about 0.5 ml of 1
mg/ml dinitrofluorobenzene (DNFB) is added for about 30
minutes. Similarly, other haptens such as and not limited
to trinitrophenyl and N-iodoacetyl-N'-(5 sulfonic 1-naphtyl)
ethylene diamine may be used. Excess DNP is removed by
20 dialyzing the membranes against about 0.15 M PBS for about
three days. The membranes are pelleted.

Alternatively, the cancer cell extract, the
peptide or the membrane, may be prepared by modifying cancer
cells of a patient with a hapten such as dinitrophenyl and
25 then preparing membranes therefrom. Cancer cells of a
patient are obtained during biopsy and frozen until needed.
About 100 mg of DNFB (Sigma Chemical Co., St. Louis, MO) was
dissolved in about 0.5 ml of 70% ethanol. About 99.5 ml of

PBS was added. DNFB concentration should be about 152

mg/0.1 ml. The solution was stirred overnight in a 37 °C

water bath. The shelf life of the solution is about 4

weeks. The cells were thawed and the pellet was resuspended

5   in 5X cells/ml in Hanks balanced salt solution. About 0.1

ml DNFB solution was added to each ml of cells and incubated

for about 30 minutes at room temperature. Similarly, other

haptens such as and not limited to trinitrophenyl and N-

iodoacetyl-N'-(5 sulfonic 1-naphtyl) ethylene diamine may be

10 used. The cells were then washed twice in Hanks balanced

salt solution. Cells are suspended in about five volumes of

about 30 mM sodium bicarbonate buffer with about 1 mM phenyl

methyl sulfonyl fluoride and disrupted with a glass

homogenizer. Residual intact cells and nuclei are removed

15 by centrifugation at about 1000 g. The membranes are

pelleted by centrifugation at 100,000 g for 90 minutes. The

membranes are resuspended in about 8% sucrose and frozen at

about -80°C until needed.

From the DNP modified cells, peptide may be

20 extracted, some of which are DNP modified as a result of

modifying the cells. Protein extraction techniques, known

to those of skill in the art, may be followed by antigen

assays to isolate antigen(s) effective for patient

treatment. The methods of isolating cell extracts are

25 readily known to those skilled in the art. Briefly,

cancerous cells are isolated from a tumor and cultured *in*

vitro. Dinitrophenyl is added to the cultured cells in

accordance with the method set forth above. Peptides are

- 27 -

isolated from cells according to an established technique of Rotzschke et al., *Nature*, 1990, *348*, 252, the disclosure of which is hereby incorporated by reference in its entirety. The cells are treated with a weak acid. Then they are
5   centrifuged and the supernatants are saved. Fractions containing small peptides are obtained by HPLC, concentrated, and frozen. The fractions are screened for immunological activity by allowing them to bind to autologous B lymphoblastoid cells which are then tested for
10 ability to stimulate melanoma-specific T lymphocytes.

The cells are treated with a weak acid, such as and not limited to trifluoroacetic acid (TFA). The cells are then centrifuged and the supernatant is saved. Compounds having a molecular weight greater than 5,000 were
15 removed from the supernatant by gel filtration (G25 Sepharose, Pharmacia). The remainder of the supernatant is separated on a reversed-phase HPLC column (Superpac Pep S, Pharmacia LKB) in 0.1% trifluoroacetic acid (TFA) using a gradient of increasing acetonitrile concentration; flow rate
20 = 1 ml/min, fraction size = 1 ml. Fractions containing small peptides are obtained by HPLC according to the method of Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), concentrated, and frozen. The fractions
25 are screened for immunological activity by allowing them to bind to autologous B lymphoblastoid cells which are then tested for ability to stimulate melanoma-specific T lymphocytes.

- 28 -

Epstein barr virus (EBV, ATCC CRL-1612, B95-8 EBV
transformed leukocytes, cotton top marmoset, *Saguinus
oedipus*) is added to B lymphoblastoid cells in culture. The
B lymphoblastoid cells are transformed into a B cell tumor
5   from the patient's own lymphocytes. Melanoma from a
metastasis is cultured in RPMI 1640 + 10% fetal calf serum
or 10% pooled human serum. The non-adhered cells are washed
off with RPMI medium. When the cells are confluent, they
are detached with 0.1% EDTA and split into two flasks. This
10   process continues where the confluent cells are continuously
split. To test for gamma interferon production by T cells,
lymphocytes from a patient's blood are obtained. The
patient's own tumor cells, which have been modified with a
hapten, such as DNP, are mixed with the lymphocytes to
15   stimulate the T cells. Every seven days, interleukin-2 is
added. The T cells are expanded by splitting a as disclosed
above. The T cells are then restimulated by the hapten
modified cells. An enriched population of T cells result
which are responsive to the hapten modified cells.

20   Human cancer vaccines have been developed and
tested by a number of workers. Although they can sometimes
induce weak immunity to a patient's cancer, they rarely
cause tumor regression. The development of inflammatory
responses in metastatic tumors was surprisingly found with
25   the DNP-vaccine of the present invention. The tumor becomes
reddened, warm and tender. Ultimately, in some cases, the
tumor regresses to the extent that the tumor disappears, to
the naked eye and microscopically. Microscopically,

infiltration of T lymphocytes into the tumor mass is

observed. Therefore, this approach, which increases the

inflammatory response and the number and capacity of

lymphocytes entering the tumor, is a significant advance in

5   the art.

The effectiveness of the vaccine may be improved

by adding various biological response modifiers. These

agents work by directly or indirectly stimulating the immune

response. Biological response modifiers of the present

10   invention include and are not limited to interleukin-12 and

gamma interferon. In this embodiment, IL12 will be given

following the each vaccine injection. Administration of

IL12 to patients with inflammatory responses is believed to

cause the T lymphocytes within the tumor mass to proliferate

15   and become more active. The increased T cell numbers and

functional capacity leads to immunological destruction of

the tumors. Dosages for IL12 will be prepared in accordance

with the dosage indications set forth above.

Patients with metastatic melanoma were treated

20   using an immunotherapy regimen with the following

components: 1) vaccine consisting of autologous tumor cells

conjugated to DNP; and 2) low dose cyclophosphamide

pretreatment. Patients were evaluated to determine whether

tumor regression had occurred, to monitor tumor inflammatory

25   responses, and to measure delayed type hypersensitivity to

autologous melanoma cells, DNFB (the form of DNP used for

skin sensitization), DNP-conjugated autologous lymphocytes,

diluent (Hanks solution), purified protein derivative (PPD),

- 30 -

and recall antigens (candida, trichophyton, and mumps).
Patients who are considered to be deriving benefit (clinical
or immunological) from the therapy are continued in the
immunotherapy regimen. Subsequent vaccines may be given
5   without cyclophosphamide. In a similar experiment,
Interleukin 2 linked to polyethylene glycol was found to not
be effective.

        In another embodiment, a vaccine comprising
chemical extracts of cancer cells conjugated to a hapten and
10 mixed with an immunological adjuvant, such as *Bacillus
Calmette-Guerin*, BCG, is used.

        In the present invention, biopsies from human
melanoma metastases were examined for expression of cytokine
mRNA using RT-PCR. mRNA for IFNγ is found in post-DNP-
15 vaccine, inflamed metastases, but only rarely in
pretreatment metastases, even those containing large numbers
of residual lymph node lymphocytes. Moreover, the Type II
cytokine, IL10, is found in almost all melanoma metastases
and appears to be produced by the melanoma cells themselves.

20   Patients with metastatic melanoma treated with an
autologous, DNP-modified vaccine develop inflammatory
responses at tumor sites. Histologically, these inflamed
lesions are characterized by T cell infiltration which is
sometimes associated with tumor cell destruction. In the
25 present invention, biopsy specimens of 8 subcutaneous
metastases that had developed inflammation following vaccine
treatment were tested for expression of mRNA for IFNγ, IL4,
TNF, and IL10. Post-vaccine, inflamed biopsies contained

- 31 -

mRNA for IFNγ (5/8), IL4 (4/8) or both (3/8), and for TNF

(4/7). In contrast, IFNγ mRNA was detected in only 1/17 and

TNF mRNA in 2/16 control specimens (pre-treatment lymph node

metastases or non-inflamed subcutaneous metastases). mRNA

5   for IL10, a cytokine with anti-inflammatory properties, was

detected in 24/25 melanoma metastases and was independent of

lymphoid content; *in situ* PCR confirmed that melanoma cells

were the major source. These findings provide a new

parameter by which to measure the effects of cancer

10   immunotherapy.

The present invention is aimed at analyzing

freshly obtained metastatic melanoma biopsies for the

presence of cytokine mRNA which correlates with a productive

immune response at the tumor site. The expression of IFNγ

15   or IL4 mRNA is characteristic of melanoma metastases that

have developed an inflammatory response following

administration of DNP-modified autologous vaccine. On the

other hand, expression of IL10 mRNA is independent of an

inflammatory response and seen in nearly all melanoma biopsy

20   specimens. Examination of cell lines derived from melanoma

biopsies as well as *in situ* PCR analysis demonstrated that

the source of IL10 is melanoma cells themselves rather than

the associated lymphocytes.

Perhaps the most important finding of this work is

25   the negative one: mRNA for IFNγ and IL4 generally is not

found in melanoma metastases from untreated patients, nor in

metastatic masses that contain large numbers of lymph node

lymphocytes. This provides a low background activity of *in*

situ cytokine production against which to compete melanoma

tissues whose T cell population has been altered by

immunotherapy.   Moreover, it underscores an important

biological point: T cells extracted from melanoma nodal

5   metastases probably represent the residua of the original

lymph node population rather than lymphocytes that have

actually infiltrated the tumor as a result of their

recognition of melanoma antigens.   Since they are not

antigen-activated, they have received no stimulus to produce

10   IFNγ or IL4.

In contrast, biopsy specimens obtained following

administration of DNP-vaccine typically expressed mRNA for

IFNγ.   However, DNP-vaccine-induced inflammatory responses

cannot be characterized as Type I since some of these

15   samples contained IL4 as well.   Given the sensitivity of

PCR-based mRNA analysis, such a pattern could be produced by

a small focus of IL4-producing T cells in the midst of a T

cell infiltrate that is predominantly IFNγ-producing.   On

the other hand, the presence of mRNA for IFNγ and IL4 could

20   signify the presence of T cells that produce both cytokines

- so-called TH$_0$ cells (Lee *et al*, *Eur. J. Immunol.* 1992

22:1455-1459).   Resolution of this issue will require

analyses that allow correlation of mRNA expression with

morphology, such as in situ PCR.   Whatever the results,

25   these findings suggest that measured of intra-tumor cytokine

production may be an important parameter to measure in

patients undergoing immunotherapy.

- 33 -

The present invention strongly suggest that the source of IL10 mRNA is the melanoma cells themselves, rather than the associated lymphocytes. Strong IL10 mRNA bands were detected in 24/25 biopsies, and its expression was

5 independent of the number of associated lymphocytes or the presence of DNP-vaccine-induced inflammation. Moreover, *in situ* PCR clearly showed IL10 mRNA within melanoma cells. Cell lines derived from the biopsy material expressed IL10 mRNA and produced IL10 as measured by ELISA.

10 The physiologic significance of IL10 production in melanoma tissues is not clear. IL10 is known to be an anti-inflammatory cytokine with ability to inhibit T cell proliferation and IL2 production (Jinquan, T., *et al.*, *J. Immunol.* 1993 *151*:4545-4551) and delayed type

15 hypersensitivity (Lee, *supra*), probably by reducing macrophage costimulatory function. Thus IL10 could suppress the activation and proliferation of melanoma-reactive T cells that have infiltrated the tumor site. However, IL10 recently has been shown to be a chemoattractant for CD8+ T

20 cells (Jinquan, *supra*); this property could account for the predominance of CD8+ cells in DNP-vaccine-induced lymphoid infiltrates. In either case, modulation of IL10 production at the tumor site may have important consequences for the tumor-host relationship.

25 The scope of the present invention also includes a method of screening for cytokine production by a tumor to determine the efficacy of an autologous, irradiated hapten conjugated cell composition in a patient suspected of having

- 34 -

cancer, said method comprising administering said hapten

conjugated composition to said patient; obtaining a sample

comprising nucleic acids from a patient tissue sample;

amplifying nucleic acids specific for a cytokine or

5 amplifying a signal generated by hybridization of a probe

specific to a cytokine specific nucleic acid in said tissue

sample; and detecting the presence of the amplified nucleic

acids or the amplified signal wherein the presence of

amplified nucleic acids or amplified signal indicates

10 cancer, wherein the presence of amplified nucleic acids or

amplified signal from said patient tissue sample indicates

efficacy of said hapten conjugated composition.

The tissue sample may be a malignant or pre-

malignant tumor, a melanoma tumor for example, or a

15 subcutaneous inflammatory metastatic melanoma, for example.

In addition, a tissue sample may be a solid or liquid tissue

sample such as and not limited to all or part of a tumor,

saliva, sputum, mucus, bone marrow, serum, blood, urine,

lymph, or a tear from a patient suspected of having cancer.

20   Nucleic acids, such as DNA (including cDNA) and

RNA (including mRNA), are obtained from the patient tissue

sample. Preferably RNA is obtained from a tissue sample.

Total RNA is extracted by any method known in the art such

as described in Sambrook et al., *Molecular Cloning: A*

25. *Laboratory Manual* (Cold Spring Harbor Laboratory, Cold

Spring Harbor, NY, 1989), incorporated herein by reference

in its entirety.

- 35 -

Nucleic acid extraction is followed by
amplification of the same by any technique known in the art.
The amplification step incudes the use of at least one
primer sequence which is complementary to a portion of a
5 cytokine specific sequence. Cytokine specific sequences are
defined for purposes of the present invention to include
(and are not limited to) all or part of sequences which
encode IFNγ, TNF, IL-2, IL-12, and IL-13. Generally, the
primer sequence is about 21 nucleotides to about 33
10 nucleotides, preferably about 21 nucleotides, about 31
nucleotides, 32 nucleotides, and about 33 nucleotides in
length.

Primer sequences useful in the amplification
methods include and are not limited to β actin, SEQ ID NOS:
15 1 and 2; IFNγ, SEQ ID NOS: 3 and 4; IL4, SEQ ID NOS: 5 and
6; IL10, SEQ ID NOS: 7 and 8; and TNF, SEQ ID NOS: 9 and 10.

Where a template dependent process of
amplification uses a pair of primers, one primer of the pair
may comprise oligonucleotides which are complementary to
20 nucleic acid sequences which encode cytokine specific
proteins. The one primer of the pair may be selected from
the group consisting of SEQ ID NOS: 1 to 10.

Alternatively, each of the two oligonucleotides in
the primer pair may be specific to a nucleic acid sequence
25 which encodes a cytokine. The primers may be designed to be
complementary to separate regions of a cytokine sequence for
example. By separate regions is meant that a first primer
is complementary to a 3' region of a cytokine sequence and a

second primer is complementary to a 5' region of a cytokine

sequence. Preferably, the primers are complementary to

distinct, separate regions and are not complementary to each

other. The primers of SEQ ID NOS: 1-10 are merely exemplary

5   of the primers which may be useful in the present invention.

         When an amplification method includes the use of

two primers, such as the polymerase chain reaction, the

first primer may be selected from the group consisting of

SEQUENCE ID NOS: 1, 3, 5, 7, and 9, and the second primer

10   may be selected from the group consisting of SEQUENCE ID

NOS: 2, 4, 6, 8, and 10. Any primer pairs which transcribe

nucleic acids toward each other and which are specific for

cytokines may be used in accordance with the methods of the

present invention.

15   Total extraction of RNA is preferably carried out.

As used herein, the term "amplification" refers to

template-dependent processes and vector-mediated propagation

which result in an increase in the concentration of a

specific nucleic acid molecule relative to its initial

20   concentration, or in an increase in the concentration of a

detectable signal. As used herein, the term

template-dependent process is intended to refer to a process

that involves the template-dependent extension of a primer

molecule. The term template dependent process refers to

25   nucleic acid synthesis of an RNA or a DNA molecule wherein

the sequence of the newly synthesized strand of nucleic acid

is dictated by the well-known rules of complementary base

pairing (see, for example, Watson, J. D. et al., In:

- 37 -

Molecular Biology of the Gene, 4th Ed., W. A. Benjamin,

Inc., Menlo Park, Calif. (1987) incorporated herein by

reference in its entirety). Typically, vector mediated

methodologies involve the introduction of the nucleic acid

5   fragment into a DNA or RNA vector, the clonal amplification

of the vector, and the recovery of the amplified nucleic

acid fragment. Examples of such methodologies are provided

by Cohen et al. (U.S. Pat. No. 4,237,224), Maniatis, T. et

al., Molecular Cloning (A Laboratory Manual), Cold Spring

10 Harbor Laboratory, 1982, each incorporated herein by

reference in its entirety.

A number of template dependent processes are

available to amplify the target sequences of interest

present in a sample. One of the best known amplification

15 methods is the polymerase chain reaction (PCR) which is

described in detail in U.S. Patents 4,683,195, 4,683,202 and

4,800,159, and in Innis et al., PCR Protocols, Academic

Press, Inc., San Diego CA, 1990, each of which is

incorporated herein by reference in its entirety. Briefly,

20 in PCR, two primer sequences are prepared which are

complementary to regions on opposite complementary strands

of the target sequence. An excess of deoxynucleoside

triphosphates are added to a reaction mixture along with a

DNA polymerase (e.g., Taq polymerase). If the target

25 sequence is present in a sample, the primers will bind to

the target and the polymerase will cause the primers to be

extended along the target sequence by adding on nucleotides.

By raising and lowering the temperature of the reaction

- 38 -

mixture, the extended primers will dissociate from the

target to form reaction products, excess primers will bind

to the target and to the reaction products and the process

is repeated. Preferably a reverse transcriptase PCR

5   amplification procedure may be performed in order to

quantify the amount of mRNA amplified. Polymerase chain

reaction methodologies are well known in the art.

Another method for amplification is the ligase

chain reaction (referred to as LCR), disclosed in EPA No.

10 320,308, incorporated herein by reference in its entirety.

In LCR, two complementary probe pairs are prepared, and in

the presence of the target sequence, each pair will bind to

opposite complementary strands of the target such that they

abut. In the presence of a ligase, the two probe pairs will

15 link to form a single unit. By temperature cycling, as in

PCR, bound ligated units dissociate from the target and then

serve as "target sequences" for ligation of excess probe

pairs. U.S. Patent 4,883,750, incorporated herein by

reference in its entirety, describes an alternative method

20 of amplification similar to LCR for binding probe pairs to a

target sequence.

Qbeta Replicase, described in PCT Application No.

PCT/US87/00880, incorporated herein by reference in its

entirety, may also be used as still another amplification

25 method in the present invention. In this method, a

replicative sequence of RNA which has a region complementary

to that of a target is added to a sample in the presence of

- 39 -

an RNA polymerase. The polymerase will copy the replicative

sequence which can then be detected.

An isothermal amplification method, in which

restriction endonucleases and ligases are used to achieve

5 the amplification of target molecules that contain

nucleotide 5'-[alpha -thio]triphosphates in one strand of a

restriction site (Walker, G. T., et al., Proc. Natl. Acad,

Sci. (U.S.A.) 1992, 89:392-396, incorporated herein by

reference in its entirety), may also be useful in the

10 amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another

method of carrying out isothermal amplification of nucleic

acids which involves multiple rounds of strand displacement

and synthesis, i.e. nick translation. A similar method,

15 called Repair Chain Reaction (RCR) is another method of

amplification which may be useful in the present invention

and is involves annealing several probes throughout a region

targeted for amplification, followed by a repair reaction in

which only two of the four bases are present. The other two

20 bases can be added as biotinylated derivatives for easy

detection. A similar approach is used in SDA.

Cytokine specific sequences can also be detected

using a cyclic probe reaction (CPR). In CPR, a probe having

a 3' and 5' sequences of non-cytokine specific DNA and

25 middle sequence of cytokine specific RNA is hybridized to

DNA which is present in a sample. Upon hybridization, the

reaction is treated with RNaseH, and the products of the

probe identified as distinctive products generating a signal

- 40 -

which are released after digestion. The original template

is annealed to another cycling probe and the reaction is

repeated. Thus, CPR involves amplifying a signal generated

by hybridization of a probe to a cytokine specific nucleic

5   acid.

Still other amplification methods described in GB

Application No. 2 202 328, and in PCT Application No.

PCT/US89/01025, each of which is incorporated herein by

reference in its entirety, may be used in accordance with

10 the present invention. In the former application,

"modified" primers are used in a PCR like, template and

enzyme dependent synthesis. The primers may be modified by

labelling with a capture moiety (e.g., biotin) and/or a

detector moiety (e.g., enzyme). In the latter application,

15 an excess of labelled probes are added to a sample. In the

presence of the target sequence, the probe binds and is

cleaved catalytically. After cleavage, the target sequence

is released intact to be bound by excess probe. Cleavage of

the labelled probe signals the presence of the target

20 sequence.

Other nucleic acid amplification procedures

include transcription-based amplification systems (TAS)

(Kwoh D., et al., Proc. Natl. Acad. Sci. (U.S.A.) 1989,

86:1173, Gingeras T. R., et al., PCT Application WO

25 88/10315, incorporated herein by reference in their

entirety), including nucleic acid sequence based

amplification (NASBA) and 3SR. In NASBA, the nucleic acids

can be prepared for amplification by standard

- 41 -

phenol/chloroform extraction, heat denaturation of a
clinical sample, treatment with lysis buffer and minispin
columns for isolation of DNA and RNA or guanidinium chloride
extraction of RNA. These amplification techniques involve

5   annealing a primer which has prostate specific sequences.
Following polymerization, DNA/RNA hybrids are digested with
RNase H while double stranded DNA molecules are heat
denatured again. In either case the single stranded DNA is
made fully double stranded by addition of second prostate

10 specific primer, followed by polymerization. The double
stranded DNA molecules are then multiply transcribed by a
polymerase such as T7 or SP6. In an isothermal cyclic
reaction, the RNAs are reverse transcribed into double
stranded DNA, and transcribed once against with a polymerase

15 such as T7 or SP6. The resulting products, whether
truncated or complete, indicate prostate cancer specific
sequences.

        Davey, C., et al., European Patent Application
Publication No. 329,822, incorporated herein by reference in

20 its entirety, disclose a nucleic acid amplification process
involving cyclically synthesizing single-stranded RNA
("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may
be used in accordance with the present invention. The ssRNA
is a first template for a first primer oligonucleotide,

25 which is elongated by reverse transcriptase (RNA-dependent
DNA polymerase). The RNA is then removed from resulting
DNA:RNA duplex by the action of ribonuclease H (RNase H, an
RNase specific for RNA in a duplex with either DNA or RNA).

- 42 -

The resultant ssDNA is a second template for a second

primer, which also includes the sequences of an RNA

polymerase promoter (exemplified by T7 RNA polymerase) 5' to

its homology to its template. This primer is then extended

5 by DNA polymerase (exemplified by the large "Klenow"

fragment of *E. coli* DNA polymerase I), resulting as a

double-stranded DNA ("dsDNA") molecule, having a sequence

identical to that of the original RNA between the primers

and having additionally, at one end, a promoter sequence.

10 This promoter sequence can be used by the appropriate RNA

polymerase to make many RNA copies of the DNA. These copies

can then re-enter the cycle leading to very swift

amplification. With proper choice of enzymes, this

amplification can be done isothermally without addition of

15 enzymes at each cycle. Because of the cyclical nature of

this process, the starting sequence can be chosen to be in

the form of either DNA or RNA.

Miller, H. I., *et al.*, PCT Application WO

89/06700, incorporated herein by reference in its entirety,

20 disclose a nucleic acid sequence amplification scheme based

on the hybridization of a promoter/primer sequence to a

target single-stranded DNA ("ssDNA") followed by

transcription of many RNA copies of the sequence. This

scheme is not cyclic; i.e. new templates are not produced

25 from the resultant RNA transcripts. Other amplification

methods include "race" disclosed by Frohman, M. A., In: *PCR*

Protocols: A Guide to Methods and Applications 1990,

Academic Press, N.Y.) and "one-sided PCR" (Ohara, O., *et*

- 43 -

al., *Proc. Natl. Acad. Sci. (U.S.A.)* 1989, *86*:5673-5677),
all references herein incorporated by reference in their
entirety.

Methods based on ligation of two (or more)
5   oligonucleotides in the presence of nucleic acid having the
sequence of the resulting "di-oligonucleotide", thereby
amplifying the di-oligonucleotide (Wu, D. Y. et al.,
Genomics 1989, 4:560, incorporated herein by reference in
its entirety), may also be used in the amplification step of
10   the present invention.

Following amplification, the presence or absence
of the amplification product may be detected. The amplified
product may be sequenced by any method known in the art,
including and not limited to the Maxam and Gilbert method,
15   see Sambrook, *supra*. The sequenced amplified product may
then be compared to results obtained with tissue excised
prior to vaccine treatment. Tissue samples obtained prior
to vaccine treatment should be free of cytokine sequences,
particularly IFNγ, TNF, IL2, IL12, and IL13. The nucleic
20   acids may be fragmented into varying sizes of discrete
fragments. For example; DNA fragments may be separated
according to molecular weight by methods such as and not
limited to electrophoresis through an agarose gel matrix.
The gels are then analyzed by Southern hybridization.
25   Briefly, DNA in the gel is transferred to a hybridization
substrate or matrix such as and not limited to a
nitrocellulose sheet and a nylon membrane. A labelled probe
is applied to the matrix under selected hybridization

- 44 -

conditions so as to hybridize with complementary DNA
localized on the matrix. The probe may be of a length
capable of forming a stable duplex. The probe may have a
size range of about 200 to about 10,000 nucleotides in

5   length, preferably about 200 nucleotides in length.
Mismatches such as and not limited to sequences with similar
hydrophobicity and hydrophilicity, will be known to those of
skill in the art once armed with the present disclosure.
Various labels for visualization or detection are known to

10   those of skill in the art, such as and not limited to
fluorescent staining, ethidium bromide staining for example,
avidin/biotin, radioactive labeling such as ^{32}P labeling, and
the like. Preferably, the product, such as the PCR product,
may be run on an agarose gel and visualized using a stain

15   such as ethidium bromide. See Sambrook et al., supra. The
matrix may then be analyzed by autoradiography to locate
particular fragments which hybridize to the probe.

          A diagnostic kit for screening for the efficacy of
an autologous, irradiated, hapten conjugated cell

20   composition comprising in one or more containers, a pair of
primers, wherein one of the primers within said pair is
complementary to a cytokine specific sequence, wherein said
primer is selected from the group consisting of SEQ ID NO:
1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5,

25   SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, and
SEQ ID NO: 10, and a means for visualizing amplified DNA;
said kit useful for determining the efficacy of said
composition.

- 45 -

The invention is further illustrated by means of
the following actual examples 1-8 and 11 and prophetic
examples 9-10 and 12-14 which are meant to be illustrations
only and are not intended to limit the present invention to
5 these specific embodiments.

Example 1

Sixty-four patients were treated with metastatic
melanoma using a melanoma vaccine, prepared in accordance
with the methods set forth above, preceded by low dose
10 cyclophosphamide (CY) and monitored for immunological
effects and anti-tumor activity. On day 0, the patients
were given cyclophosphamide 300 mg/M^2 i.v. Three days later,
they were injected intradermally with vaccine consisting of
10 x 10^6 to 25 x 10^6 autologous, cryopreserved, irradiated
15 (2500 R) tumor cells mixed with BCG; the tumor cells were
obtained by dissociation of metastatic masses enzymatically
(collagenase and DNAse). This treatment sequence was
repeated every 28 days for 8 treatments.

The toxicity of the therapy was limited to a local
20 inflammatory response at the injection site and mild nausea
and vomiting following cyclophosphamide administration.
There were 40 evaluable patients with measurable metastases;
5 had responses - 4 complete and 1 partial. The median
duration of response was 10 months (7-84 + months). One
25 patient continues to be in remission at 11 years.
Regression occurred not only in skin and nodal metastases,
but also in lung and liver metastases. In 6 additional

- 46 -

patients, an anti-tumor response was observed that seemed
peculiar to this vaccine therapy, i.e., the regression of
metastatic lesions that appeared after the immunotherapy was
begun. In 3 patients this "delayed" regression occurred in
5   two or more tumors.

Delayed-type hypersensitivity (DTH) to autologous,
mechanically-dissociated melanoma cells was detectable in
only 16% of patients before treatment, as compared with in
46%, 56% and 73% of patients on days 49, 161 and 217,
10 respectively. The increases in delayed type
hypersensitivity following immunotherapy were statistically
significant by a non-independent t-test; day 0 vs. day 49, p
< 0.001; day 0 vs. day 161, p < 0.001; day 0 vs. day 217, p
= 0.021. Overall, 26/43 patients (61%) exhibited a positive
15 delayed type hypersensitivity response ($\geq$ 5 mm) to
autologous melanoma cells at some time point. Patients also
developed strong delayed type hypersensitivity to the
enzymes used to prepare the tumor cell suspensions: of 24
patients tested for delayed type hypersensitivity with a
20 mixture of collagenase and DNase (each at 1 μg/ml) after two
vaccine treatments, 21 (88%) had responses > 5mm. Anti-
tumor responses to the vaccine were strongly associated with
delayed type hypersensitivity to mechanically-dissociated,
autologous melanoma cells, as indicated by three
25 observations: 1) 8/10 patients who exhibited tumor
regression had positive delayed type hypersensitivity; 2) in
post-surgical adjuvant patients, there was a highly
significant correlation between the intensity of delayed

type hypersensitivity to autologous melanoma cells and the time to recurrence of tumor ($r = 0.680$, $p < 0.001$); 3) nine patients who developed delayed type hypersensitivity to the autologous melanoma cells in their original vaccine ("old"

5   tumor) developed new metastases ("new" tumor) that did not elicit delayed type hypersensitivity or elicited a much smaller response. The patients were compared to their condition prior to treatment with the vaccine. The patients treated prior to the vaccine study were removed from

10   treatment one to two months prior to starting the vaccine study. Accordingly, the patients were untreated beginning the vaccine study.

         In three cases we were able to excise regressing tumors for histological examination; such tumors were

15   characterized by an intense infiltration of lymphocytes. In contrast, tumors excised from these patients before immunotherapy consisted of homogeneous masses of malignant cells without significant lymphocytic infiltration.

         This study shows that the use of cyclophosphamide

20   allows the development of an immune response to melanoma-associated antigens in cancer-bearing patients.

Example 2

         Patients with metastatic melanoma were sensitized to DNP by topical application to the upper arm with 1%

25   dinitrochlorobenzene (DNCB) or dinitrofluorobenzene (DNFB). Two weeks later they were injected with a vaccine consisting of 10×10^6 to 25×10^5 autologous, irradiated melanoma cells

- 48 -

conjugated to DNP and mixed with BCG. Cyclophosphamide 300

mg/M² i.v. was given 3 days before DNCB (or DNFB) or vaccine.

Of 4 evaluable patients, 3 have developed a striking

inflammatory response in tumor masses after 2 vaccine

5   treatments (8 weeks). Patient #1 developed erythema and

swelling in the > 50 large (1-3 cm) dermal metastases on her

leg and lower abdomen, followed by ulceration and drainage

of necrotic material, and some are beginning to regress.

Biopsy showed infiltration with CD4+ CD8+ T lymphocytes.

10 Patient #2 developed erythema and swelling in the skin of

her lower abdomen and groin overlying large (8 cm) nodal

masses. These have not yet regressed, but have changed in

consistency from rockhard to fluctuant. Patient #3

exhibited moderate erythema in the skin overlying 3

15 subcutaneous metastases. All 3 patients have developed

delayed type hypersensitivity to both DNCB and to DNP

conjugated autologous lymphocytes.

The patients were compared to their condition prior to

treatment with the vaccine. The patients treated prior to

20 the vaccine study were removed from treatment one to two

months prior to starting the vaccine study. Accordingly,

the patients were untreated beginning the vaccine study.

Example 3

Fifteen patients (including 3 patients from

25 Example 2) were treated with metastatic melanoma using a

novel form of immunotherapy, i.e., tumor cell vaccine

conjugated to DNP. Patients were sensitized to DNP by

- 49 -

topical application to the upper arm with 5%

dinitrochlorobenzene. Then every 4 weeks they received

cyclophosphamide 300 mg/M^2 followed 3 days later by injection

of 10 x 10^5 to 25 x 10^5 autologous, irradiated melanoma cells

5   conjugated to DNP. Patients received 6-8 treatments. Most

patients (92%) developed delayed-type hypersensitivity (DTH)

to DNP-conjugated autologous lymphocytes or tumor cells

(mean DTH = 17mm). The vaccine induced a striking

inflammatory response in subcutaneous and nodal metastases

10   in 11/15 patients, consisting of erythema, swelling, warmth,

and tenderness around tumor masses, and, in one case,

purulent drainage. Biopsies showed infiltration with

lymphocytes, which, by immunopathological and flow

cytometric analyses, were mainly CD3+, CD4-, CD8+, HLA-DR+ T

15   cells. The melanoma cells in these tissues strongly

expressed ICAM-1, which serves as an adhesion molecule for T

cells. Thus, DNP-vaccine seems to induce a degree of anti-

melanoma immunity not seen with previously tested

immunotherapy. The patients were compared to their

20   condition prior to treatment with the vaccine. The patients

treated prior to the vaccine study were removed from

treatment one to two months prior to starting the vaccine

study. Accordingly, the patients were untreated beginning

the vaccine study.

25   Example 4

         This example examined the therapeutic effects of

DNP-vaccine in patients with surgically-resected metastases

- 50 -

and no clinical evidence of metastatic disease.   Forty seven

patients were sensitized to the hapten, DNFB

(dinitrofluorobenzene).   Then they were treated by

intradermal injection of autologous, irradiated melanoma

5   cells conjugated to DNP.   Additional vaccine injections were

administered ever 28 days for a total of eight treatments.

All patients were periodically tested for Delayed Type

Hypersensitivity, DTH, responses to autologous melanoma

cells, DNP-conjugated autologous lymphocytes, and microbial

10   antigens.   *In vitro* studies were performed with

cryopreserved lymphocytes extracted from metastatic tumors

and/or separated from peripheral blood.

The graph of Figure 12 compares the percent cf

patients tumor free in the months following surgery treated

15   with DNP vaccine and non-haptenized control vaccine.   The

study examined the therapeutic effects of DNP-vaccine in

patients with surgically-resected metastases and no clinical

evidence of metastatic disease.   All patients were

sensitized to the hapten, DNFB (dinitrofluorobenzene).   Then

20   they were treated by intradermal injection of autologous,

irradiated melanoma cells conjugated to DNP.   Additional

vaccine injections were administered over 28 days for a

total of eight treatments.   All patients were periodically

tested for Delayed Type Hypersensitivity, DTH, responses to

25   autologous melanoma cells, DNP-conjugated autologous

lymphocytes, and microbial antigens.   *In vitro* studies were

performed with cryopreserved lymphocytes extracted from

metastatic tumors and/or separated from peripheral blood.

- 51 -

DNP-VACCINE THERAPY OF MELANOMA

DAY OF WEEK	M	W	F	M	T	M	W	TH	M	TH	M	W	M
DAY OF STUDY	-21	-19	-17	-14	-13	0	2	3	28	31	49	51	56
CYCLO PHOSPH AMIDE			X			X			X				REPEAT
DNP VACCINE								X		X			CYCLE
DNFB SENS				X	X								FOR
DNFB CHALL						X							A
APPLY SKIN TESTS	X					X					X		TOTAL
READ SKIN TESTS		X					X					X	OF
OBTAIN PBL	X					X					X		8
OBTAIN SERUM	X					X					X		VACCINES
ROUTINE LABS	X										X		

CY (cyclophosphamide) = 300 mg/M^2 i.v. bolus only before
first two vaccines

VACCINE = 5 x 10^6 to 20 x 10^6 autologous, irradiated melanoma
cells mixed with BCG

DNFB SENS = 1.0 mg in 0.1 ml acetone-corn-oil applied to
ventral upper arm

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- 52 -

DNFB CHALL = 200 μg in 0.1 ml acetone-corn-oil applied to

forearm

APPLY SKIN TESTS = autologous melanoma cells, peripheral

blood lymphocytes (PBL), peripheral blood lymphocytes

5   conjugated to DNP (PBL-DNP), purified protein derivative

(PPD) (skin test for tuberculosis), microbial recall

antigens

*Day 0: PBL, PBL-DNP only

READ SKIN TESTS = mean diameter of induration

10   OBTAIN PBL = 100 cc heparinized blood

ROUTINE LABS = complete blood count (CBC), differential

blood count (diff), platelet count (platelets), SMA-12

(panel of routine lab tests, blood urea nitrogen (BUN)

Sensitization to DNP - Patients initially were

15   sensitized to DNP as follows: On day -17, cyclophosphamide,

300 mg/M^2, was administered as a rapid i.v. infusion. Three

days later, on days -14 and -15, patients were sensitized

with DNFB (dinitrofluorobenzene): 1 mg DNFB dissolved in

acetone-corn oil and applied topically in a volume of 0.1 ml

20   within the confines of a 2 cm diameter steel ring. Two

weeks later, patients were tested for reactivity to DNP by

topical application of 200 μg DNFB and intradermal injection

of DNP-conjugated autologous PBL. Cyclophosphamide was

reconstituted in sterile water and the proper dosage was

25   administered by rapid i.v. infusion.

Vaccine Preparation - Tumor masses were processed.

Cells were extracted by enzymatic dissociation with

collagenase and DNAse and by mechanical dissociation, frozen

in a controlled rate freezer, and stored in liquid nitrogen

until needed. On the day that a patient was to be treated,

the cells were thawed, washed, and irradiated to 2500 R.

5 Then they were washed again and suspended in Hanks balanced

salt solution without phenol red.

Conjugation of melanoma cells with DNP was

performed. This involved a 30 minute incubation of tumor

cells with dinitrofluorobenzene (DNFB) under sterile

10 conditions, followed by washing with sterile saline.

The vaccine consists of 5-20 x 10^6 live tumor cells

suspended in 0.2 ml Hanks solution. When BCG is added, it

consisted of 0.1 ml of a 1:10 dilution of Tice BCG. Each

vaccine treatment consisted of three injections into

15 contiguous sites on the upper arms or legs, excluding limbs

ipsilateral to a lymph node dissection.

Study Procedure - On day 0, patients received

cyclophosphamide 300 mg/M^2 as a rapid i.v. infusion. Three

days later, on day +3, they were injected intradermally with

20 autologous melanoma vaccine. Additional vaccine injections

were administered every·four weeks for a total of eight

treatments. Cyclophosphamide was given only prior to the

first two injections. All vaccines were DNP-conjugated and

mixed with Bacillus Calmette-Guerin (BCG). BCG is the Tice

25 strain (substrain of the Pasteur Institute strain) obtained

from Organon Teknika Corporation, Durham, NC. The freeze-

dried material was reconstituted with 1 ml sterile water and

diluted 1:10 in phosphate-buffered saline, pH 7.2; then 0.1

- 54 -

ml was drawn up, mixed with the vaccine and injected. All
vaccines were injected into the same site (upper arm or
leg).

    <u>Immunological evaluation</u> - Skin-testing was
5 performed by the intradermal injection of 0.1 ml of test
material on the forearm, and delayed type hypersensitivity
was assessed at 48 hours by measuring the mean diameter of
induration. Positive reactions were photographed. The
following materials were tested: 1) 1×10^6 irradiated
10 autologous melanoma cells; 2) 3×10^5 autologous peripheral
blood lymphocytes, both unconjugated and conjugated to DNP;
3) Hanks solution; 4) PPD-intermediate strength; and 5)
microbial recall antigens - Candida, trichophyton, and
mumps. Also, contact sensitivity to DNFB was tested by
15 applying 200 μg to the skin of the forearm and examining the
area for a circle of induration at 48 hours.

    All patients had blood collected for separation
and cryopreservation of lymphocytes and serum each time
skin-testing was performed (see Table 1 for schedule of
20 blood drawing). Periodically, these were tested for: 1)
proliferative and cytotoxic response to autologous melanoma
cells; and 2) proliferative response to DNP-conjugated
autologous lymphocytes.

- 55 -

<u>Duration of Study</u>

1) Patients were treated with eight courses of vaccine which required about eight months. Treatment was then stopped. These patients will be monitored until at
5 least five years has elapsed since their initial surgery.

2) Patients who developed regional recurrence or distant metastases before the completion of eight treatments were taken off the study and treated as clinically indicated (chemotherapy or surgery).

10   The control group consisted of 22 patients with melanoma metastatic to regional lymph nodes. They underwent surgical resection of their disease, at which time they had no clinical evidence of metastatic melanoma. Then, they received treatment with a non-haptenized, autologous
15 melanoma vaccine. First, they were given cyclophosphamide, 300 mg/M². Three days later they were injected intradermally with the vaccine, which consisted of 10 x 10⁶ to 25 x 10⁵ irradiated, autologous melanoma cells mixed with BCG. The cyclophosphamide-vaccine treatment was repeated every 28
20 days. A total of eight treatments was given. The patients were clinically evaluated every two months.

Only 20% of the control patients were cancer-free at two years. In contrast, patients treated with the DNP-vaccine of the invention had significantly higher cancer-
25 free survival as set forth above.

The patients who received haptenized vaccine all had melanoma metastatic to regional lymph nodes, but no evidence of distant metastases. Patients in this condition

- 56 -

are routinely treated by surgical resection of the diseased

nodes. Surgical resection renders them clinically disease-

free, but they have an 80-85% chance of developing

metastatic melanoma with two years.

5   The patients in the control group were in the same

clinical condition in order to be comparable to the

haptenized vaccine group. Thus, the control group also

consisted of patients with melanoma metastatic to regional

lymph nodes, but no evidence of distant metastases, who had

10 undergone surgical resection of the diseased nodes. When

treatment was initiated with the non-haptenized vaccine, the

control patients were clinically disease-free, but as

previously noted, 80% developed distant metastases.

           Patients with surgically incurable melanoma were

15 not selected as controls because such patients have a cure

rate approaching zero, and an even shorter survival than

patients with resectable lymph node metastases. Moreover,

it is not possible in such patients to measure disease-free

survival, a parameter that was dramatically prolonged by the

20 vaccine of the present invention.

           A statistical analysis of the data was performed

as follows: Kaplan-Meir plots of disease-free survival and

total survival were constructed. The difference between

DNP-vaccine patients and control patients was analyzed by

25 the Mantel log-rank test. These are standard statistical

methods for analyzing such data. The difference was highly

significant with $p < .01$.

- 57 -

Seventeen patients additional were subsequently treated according to the protocol outlined above (the size of the control group was not increased for reasons set forth above). The results maintained statistically significant

5   differences in disease-free survival and total survival.

Example 5

Administration of an autologous, dinitrophenyl (DNP)-conjugated melanoma vaccine induces T cell infiltration of metastatic tumors, and prolongs survival of

10 patients who have undergone lymphadenectomy for bulky regional metastases. These effects appear to be due to melanoma-specific T cells. Their generation is contingent upon T cells with specificity for DNP-modified melanoma cells (DNP-MEL).

15 Clinical Protocol

All patients had metastatic melanoma and were undergoing immunotherapy with autologous, DNP-conjugated tumor vaccine, as previously described in Berd, D., et al., *Cancer Res.*, 1991, *51*, 273 I, incorporated herein by

20 reference in its entirety. Informed consent was obtained from the patients. Patients were pre-treated with cyclophosphamide 300 mg/M^2, see Berd et al. (1986) *supra*, and three days later were sensitized to DNFB by topical application of 0.1 ml of a 1 DNFB solution in acetone-corn

25 oil on two consecutive days. Two weeks later patients were again given cyclophosphamide, followed 3 days later by

- 58 -

injection of DNP-conjugated melanoma vaccine. DNP-vaccine

was repeated every 28 days. Cyclophosphamide was given

prior to the first two cycles. The vaccine consisted of 10

x 10^5 - 25 x 10^5 cryopreserved, autologous, irradiated (2500

5   R), DNP-conjugated melanoma cells conjugated to DNP mixed

with BCG. All tumor preparations contained lymphocytes

which were the residua of tumor-infiltrated lymph node

tissue. Serum and PBL were collected at the following time

points: day 0 (before sensitization), day 14 (2 weeks after

10 DNFB sensitization), day 63 (after 2 vaccines), day 119

(after 4 vaccines), day 175 (after 6 vaccines), and day 231

(after 8 vaccines).

<u>Cellular Reagents</u>

PBL were separated by density gradient

15 centrifugation on Ficoll metrizoate. They were suspended in

freezing medium (RPMI-1640 (Mediatech, Washington DC) + 1%

human albumin + 10 dimethyl sulfoxide) frozen in a

controlled-rate freezer, and stored in liquid nitrogen. HLA

typing of PBL was performed by the Thomas Jefferson

20 University Hospital Clinical Laboratory.

Melanoma cells were enzymatically extracted from

metastatic masses according to the method of Berd, D., et

al., (1986) *supra*, incorporated herein by reference in its

entirety, and cryopreserved. Cell lines were derived from

25 these suspensions and were maintained in RPMI-1640 with 10%

fetal calf serum. Melanoma cell lines from the patients

used in this study were distinguished by MHC class I

differences determined by flow cytometric analysis with a panel of monoclonal antibodies obtained from the American Type Culture Collection: (HB82 = HLA-A2, HB122 = HLA-A3, HB164 = HLA-A11,24).

5 Hapten Conjugation

PBL were DNP-modified by a 30 minute incubation with aqueous DNFB or DNBS, according to the methods of Miller, S. D. and H. N. Claman, *J. Immunol.*, 1976, *117*, 1519 and Geczy, A. F. and A. Baumgarten, *Immunology*, 1970, *19*, 10 189, (incorporated herein by reference in their entirety) respectively; the two methods yielded equivalent results. For specificity controls, cells were modified with TNP by incubation with TNBS, or with oxazolone, according to the methods of Fujiwara, H., et al., *J. Immunol.*, 1980, *124*, 863 15 and Boerrigter, G. H. and R. J. Scheper, *J. Invest. Dermatol.*, 1987, *88*, 3, (incorporated herein by reference in their entirety) respectively. Hapten conjugation was repeated with melanoma cells.

Delayed-Type Hypersensitivity (DTH)

20   Cryopreserved PBL were thawed, washed, and resuspended in Hanks balanced salt solution. The cells were divided into three groups: unmodified, conjugated to DNP, and conjugated to TNP. After washing, 1×10^6 melanoma cells or 3×10^6 PBL were suspended in 0.1 ml Hanks solution and 25 injected intradermally on the forearm. DTH was determined

- 60 -

at 48 hours by measuring the mean diameter of induration. The DTH assay was repeated with melanoma cells.

All patients developed DTH to DNP-modified autologous PBL (Figure 1). DTH responses were evident two

5 weeks after topical application of DNFB (day 14), and then remained stable throughout the period of monthly vaccine administration. DNP-conjugated autologous melanoma cell suspensions elicited stronger DTH than DNP-PBL (mean SE: PBL = 13.3 mm ± 1.3 mm, melanoma cells = 21.9 mm ± 3.6 mm; p <

10 0.01). DTH was specific for DNP-modified "self", since autologous PBL conjugated to TNP elicited no response in 50 patients tested.

Anti-DNP Antibody

An ELISA was developed by coating microtiter wells

15 with DNP-conjugated PBL. This method was found to be preferable to coating plates with DNP-conjugated albumin because it resulted in lower background readings with serum of pre-immunized patients. DNP-conjugated PBL (5 x 10^5 in 0.1 ml) were added to each well of 96 flat bottom plate.

20 The cells were fixed to the plate by drying followed by a 5 minute exposure to 100% methanol. Then, the plates were washed five times with phosphate buffered saline + 0.05 % Tween-20. Serial dilutions of test sera were added to the wells and the plate was incubated in a humidified chamber at

25 37° C for 1 hour. After the incubation, the plate was washed five times, and then horse radish peroxidase-conjugated goat anti-human immunoglobulin (Cappel

- 61 -

Laboratories, Malvern, PA) was added at predetermined

optimal dilution. For detecting IgG or IgM antibodies,

peroxidase-conjugated goat anti-human IgG or IgM were used,

respectively. After a 1 hour incubation at 37° C, the plate

5   was washed five times and 0.1 ml of substrate (O-

phenylenediamine, Sigma Chemical Co., St. Louis, MO) was

added to each well followed by 50 μl of 0.12 % hydrogen

peroxide. The plate was read in an ELISA plate reader.

The assay was validated using a murine anti-DNP

10 monoclonal antibody (clone SPE-7; Sigma ImmunoChemicals) and

a peroxidase-conjugated antimouse immunoglobulin antibody as

the second step reagent. Subsequently, the positive control

consisted of a serum sample from a patient who had received

multiple injections of DNP-vaccine. Anti-DNP antibody titer

15 of each serum sample was defined as follows:

(peak OD of sample) X (reciprocal of the dilution having an

OD equal to half the peak OD of positive control)

Butler, J. E., *Methods Enzymol.*, 1981, 73, 482.

Anti-DNP antibody developed in 24 out of 27

20 patients tested (Figure 2). In contrast to DTH, antibody

was not induced by DNFB topical application (day 14). In 19

patients, titers increased above pre-immunization levels

after two intradermal injections of DNP-conjugated melanoma

cells (day 63); in 5 additional patients, significant titers

25 were found only after 4 to 6 vaccines. In all patients, IgG

antibody was detected; anti-DNP IgM was found in only three

patients. Anti-DNP antibody cross reacted with TNP, shown

- 62 -

by binding to TNP-modified cells, but not to the unrelated

hapten, oxazolone.

Development of T cell Lines

          PBL (1 x 10⁶) were mixed with autologous DNP-

5 conjugated B lymphoblastoid cells (1 x 10⁵) in 24 well flat

bottom plates in lymphocyte culture medium. After 7 days of

culture, IL2 100 U/ml (a gift of Cetus Oncology, Emeryville,

CA) was added. Expanding T cell cultures were maintained in

medium + IL2 and were split as needed to maintain a

10 concentration of about 2 x 10⁶ cells in a 22 mm diameter

well. Every 14 days, the cultures were restimulated by

adding autologous DNP-conjugated B lymphoblastoid cells.

Phenotypes were determined by flow cytometry with a panel of

monoclonal antibodies (Becton-Dickinson, San Jose, CA).

15 Separation of CD8+ and CD4+ T cells was accomplished by

indirect panning in which T cells coated with anti-CD8 or

anti-CD4 monoclonal antibodies were adhered to anti-

immunoglobulin-coated dishes using standard techniques

according to the methods of Wysocki, L. J. and V. L. Sato,

20 *Proc. Natl. Acad. Sci. USA*, 1978, 75, 2844, incorporated

herein by reference in its entirety; the adherent cells were

isolated and expanded with DNP-modified stimulators and IL2.

          Phenotypically homogeneous subpopulations of T

cells were obtained by culturing at limiting dilution in

25 round-bottom microtiter wells in lymphocyte culture medium

containing 2 x 10⁵ irradiated allogeneic feeder cells, 200

U/ml IL2, and phytohemagglutinin. Wells with growing

- 63 -

lymphocyte colonies were screened for ability to proliferate

in response to DNP-modified B lymphoblastoid cells.

Positive wells were expanded in IL2 and restimulated with

autologous DNP-conjugated B lymphoblastoid cells every 14

5 days.

Lymphoproliferative Responses - PBL were tested as responder

cells. They were suspended in lymphocyte culture medium

(RPMI-1640, 10% pooled human AB⁻ serum, insulin-transferrin-

selenite media supplement (Sigma Chemical Co.) 2 mM L-

10 glutamine, 1% non-essential amino acids, 25 mM HEPES buffer,

penicillin + streptomycin) and added to 96-well, round

bottom microtiter plates at 1 x 10^5 cells/well. Stimulator

cells included: 1) autologous or allogeneic PBL, 2)

autologous or allogeneic B lymphoblastoid lines made by

15 transfection with Epstein-Barr virus, 3) autologous cultured

melanoma cells; they were inactivated by irradiation (5000

R). In most experiments, the responder:stimulator ratio was

1:1. The plates were incubated in a CO_2 incubator at 37°C

for 5 days; then the wells were pulsed with ^{125}I-labeled IUDR

20 (ICN Radiochemical, Costa Mesa, CA) for 6 hours, harvested

with an automatic harvesting device, and counted in a gamma

counter. The mean of triplicate wells was calculated.

Cultured T cells were also tested for a lymphoproliferative

response in accordance with the above methods.

25   PBL, obtained and cryopreserved from four patients

at the time of maximum DTH reactivity to DNP-modified

autologous cells, were thawed and tested for in vitro

- 64 -

proliferative responses. PBL from all four patients
proliferated upon stimulation with DNP-modified cells
(Figure 3). The kinetics of the development of the
proliferative response in one of these patients (DM2) is

5   shown in Figure 4. DNFB application alone (day 14) did not
result in detectable numbers of circulating responding
cells. Reactive PBL were detected after two injections of
DNP-vaccine (day 63) and continued to be detected throughout
the 8 months period of vaccine treatment.

10   The proliferative response to DNP-modified cells
was specific, since neither unconjugated PBL nor PBL
modified with TNP evoked responses (Figure 5). Post-vaccine
PBL also proliferated briskly when stimulated with a DNP-
modified melanoma cell line derived from autologous tumor

15   tissue. When stimulated with allogeneic lymphocytes, PBL
exhibited the expected mixed lymphocyte reaction which was
three to five-fold greater than the DNP responses.

            Circulating T lymphocytes from one of these
patients (DM2) were expanded *in vitro* by culture in IL2 and

20   repeated restimulation with autologous DNP-modified B
lymphoblastoid cells. After four weeks of expansion, the T
cells were 70% CD3+, CD8+ and 30% CD3+, CD4+. They
proliferated when stimulated by autologous, DNP-modified B
lymphoblastoid cells or DNP-modified, cultured melanoma

25   cells, but not by unconjugated autologous cells (Figure 6).
These cells were separated by positive panning into CD8-
enriched and CD4-enriched populations that were 98% pure as
determined by flow cytometry analysis. As shown in Figure

- 65 -

7, both CD4-enriched and CD8-enriched T cells exhibited a
proliferative response to DNP-modified autologous B
lymphoblastoid cells. However, only CD8+ T cells responded
to DNP-modified autologous melanoma cells. This result may
5 have been due to the low constitutive expression (< 5%) of
MHC class II by the melanoma cell line.

Expanded T cells were tested for ability to
produce cytokines when stimulated with autologous, DNP-
modified B lymphoblastoid cells. As shown in Figure 8, they
10 produced gamma interferon but not IL4. To determine whether
both CD4+ and CD8+ T cells were involved in the cytokine
response, sublines that were obtained by plating T cells at
limiting dilution were analyzed. Each of these cultures was
homogeneous in respect to expression of CD4 and CD8. Three
15 of these sublines (two CD4+, one CD8+) were tested for
cytokine response to DNP-modified B lymphoblastoid cells.
All three produced gamma interferon, while none made IL4
(Figure 8).

20 <u>Cytokine Production</u> - T cells were added to round bottom
microtiter plates at 1 x 10^5 cells/well. An equal number of
stimulators (DNP-modified autologous B lymphoblastoid cells)
was added, and supernatants were collected after 18 hours
incubation. Commercially available ELISA kits were used to
25 measure gamma interferon (Endogen, Boston, MA; sensitivity =
5 pg/ml) and IL4 (R&D Systems, Minneapolis, MN; sensitivity
= 3 pg/ml).

- 66 -

To determine the MHC-dependence of the response, stimulator cells were pre-incubated with monoclonal antibodies to MHC class I (W6/32) or MHC class II (L243) at a concentration of 10 μg/ml for one hour before adding
5   responder cells. Non-specific mouse immunoglobulin at the same concentration was tested as a negative control.

DNP-reactive CD8+ T cells obtained by panning of the bulk population were able to be maintained in long-term (> 3 months) culture in IL2-containing medium by repeated
10   stimulation with DNP-modified autologous B lymphoblastoid cells; they retained the stable phenotype, CD3+, CD8+. Two lines of evidence confirmed that their response was MHC class I restricted: 1) Gamma interferon production was blocked by pre-incubation of stimulator cells with anti-
15   class I framework antibody, but not by anti-class II antibody (Figure 9), 2) The T cells were able to respond to allogeneic DNP-modified stimulators that were matched at one or both HLA-A loci, but not to stimulators that were HLA-A mismatched. As shown in Figure 10, T cells proliferated
20   upon stimulation with DNP-modified autologous PBL (HLA-A1, A2, B8+, Bw6) and with DNP-modified allogeneic PBL that expressed A1 or A2 or both; no response was elicited by DNP-modified allogeneic stimulators that were A1 and A2-negative.

25   Cytotoxicity - Melanoma targets were labeled for two hours with ^{51}Cr (Amersham Corp, Arlington Heights, IL), and 2500 cells were added to round-bottom microtiter wells. Then

- 67 -

effector cells were added to achieve a series of E:T ratios.
After 6 hours incubation at 37° C supernatants were removed
and counted in a gamma counter. Lysis was defined as:

$([CPM_{test} - CPM_{spontaneous}] / [CPM_{total} - CPM_{spontaneous}]) * 100.$

5   The cytotoxicity of the CD8+ T cell line was
tested in a ^{51}Cr-release assay with autologous melanoma cells
as targets. To minimize spontaneous ^{51}Cr release, DNP
modification was accomplished with DNBS rather than DNFB. T
cells lysed DNP-modified autologous melanoma cells but not
10 allogeneic (class I-mismatched) melanoma cells (Figures 11a,
11b). There was a direct relationship between
susceptibility to lysis and the degree of DNP modification,
as determined by the concentration of DNBS used. Neither
autologous nor allogeneic targets modified with TNP were
15 lysed.

Example 6

          Clinical data was collected to suggest that an
autologous, DNP-conjugated melanoma vaccine prolongs
20 disease-free survival (DFS) and total survival (TS) in
melanoma patients with bulky but resectable regional lymph
node metastases. Forty-seven patients underwent standard
lymphadenectomy with resection of metastatic masses. Tumor
cells were enzymatically-dissociated from these tissues and
25 cryopreserved. Vaccines consisted of 10 x 10^6 to 20 x 10^6
irradiated (2500 cGy) melanoma cells, conjugated to DNP and
mixed with BCG. They were injected i.d. every 28 days for a
total of 8 treatments. Cyclophosphamide 300 mg/M^2 i.v. was

- 68 -

given 3 days before the first 2 vaccines only. The DFS and

TS of these patients were compared with those of 22 melanoma

patients with resected nodal metastases treated previously

with an unconjugated vaccine, see Example 4. Of 36 patients

5 with stage 3 melanoma (palpable mass in one lymph node

region), 22 are disease-free with a median follow-up of 33

months. Kaplan-Meir analysis projects a 3 year DFS and TS

of 59% and 71%, respectively. In contrast, the DFS and TS

of stage 3 patients treated with unconjugated vaccine was

10 22% and 27% respectively (p = 0.01, log-rank test). Of 11

stage 4 patients (palpable mass in two lymph node regions),

5 are NED (no evidence of disease) with a median follow-up

of 41 months. For both stage 3 and 4 patients, the highest

rate of relapse was in the first 6 months, a time when anti-

15 melanoma immunity might not have yet been established. This

experiment will be followed by an accelerated schedule of

immunizations to reduce the rate of early relapses and

improve the overall clinical outcome. The patients were

compared to their condition prior to treatment with the

20 vaccine. The patients treated prior to the vaccine study

were removed from treatment one to two months prior to

starting the vaccine study. Accordingly, the patients were

untreated beginning the vaccine study.

Example 7

25 Materials and Methods

Human melanoma tissue and cell lines - Tissue was obtained

from patients with metastatic melanoma prior to entry into

the vaccine program and at post vaccine time points. The

clinical protocol for the DNP-vaccine administration was

performed in accordance with Berd, D., et al., Cancer Res.

1991 51:2731-2734. Following surgery, the tumor specimen

5   was transported to the laboratory, tumor tissue was isolated

from surrounding fascia and connective tissue, and pieces of

tumor measuring 2-4 mm^3 were snap frozen in liquid nitrogen.

Melanoma cell lines from the same specimens were derived

from enzyme digests (DNAase and collagenase) of the tumor

10 and propagated as described by Berd, D., et al. Cancer Res.

1986 46:2572-2577.

Isolation of RNA and Amplification via RT-PCR - Total RNA

was extracted from frozen tissues by grinding in guanidium

isothiocyanate, followed by isolation using CsCl gradient as

15 described by Lattime, E.C., et al., J. Immunol. 1988

144:3422-3428. To minimize the loss of tissue RNA, 15 μg of

E. coli ribosomal RNA (Sigma Chemical Corp., St. Louis, MO)

was added to each sample. Isolated RNA was resuspended in

diethylpyrocarbonate treated (DEPC-tx) (Sigma) deionized

20 distilled water. cDNA synthesis was performed using 10 ug

total RNA, Random Primer (Gibco BRL, Gaithersburg, MD), and

RT buffer in DEPC-tx water. This was incubated at 65°C for

10 min and then placed at 4°C. To this, 10 mM DTT (Gibco

BRL), 0.5 mM each dATP, dCTP, dTTP, dTTP, (Gibco BRL),and

25 500 U MMLV-RT (Gibco BRL) was added to achieve a final

reaction volume of 50 μl. Samples were incubated at 37°C

for 1 hour, then heated to 95°C for 5 min.

- 70 -

For amplification by PCR, 5 ul of each cDNA was
then added to MicroAmp reaction tubes (Perkin Elmer,
Norwalk, CT) containing PCR Reaction buffer, 0.2 mM each
dATP, dCTP, dGTP, dTTP, 1.25 U AmpliTaq DNA Polymerase

5 (Perkin Elmer), MgCl$_2$ concentrations determined to be optimal
for each primer pair (final concentrations of 1.5-6.0 mM),
and 0.5 mM each of the appropriate primer pairs in a final
volume of 50 μl. Primer pairs used in this study included
β-actin, TNF-α, IL-4, IFNγ, South San Francisco, CA) and

10 IL10 (Clontech, Palo Alto, CA). β-actin served for a
standard for comparison of relative mRNA expression between
samples, as well as a control for RT and PCR reactions. PCR
samples were amplified using a GeneAmp System 9600
thermocycler Perkin Elmer). Each sample was denatured at

15 94°C for 37 sec, annealed at 55°C for 45 sec, and extended
at 72°C for 60 sec for 39 cycles, followed by a 10 min.
extension at 72°C.

PCR products and size markers (Novagen, Madison,
WI) were separated in a 2.0% agarose gel (FMC BioProducts,

20 Rockland, ME). The gel was stained with ethidium bromide,
visualized, and photographed under UV illumination.
Electrophoresis of PCR products revealed a band
corresponding to the predicted fragment size for each set of
primers. Nonreverse transcribed RNA was subjected to

25 amplification by PCR as a control for genomic DNA
contamination.

Cryopreserved, enzyme-dissociated cell suspensions
of melanoma tissues were found not to be suitable for RNA

analysis- These samples usually expressed mRNA for all cytokines tested, probably a result of activation by the dissociation process.

Histology and In-Situ RT-PCR - Routine H&E staining of

5   representative specimens was done by the Department of Pathology. *In situ* RT-PCR was done on paraffin sections which were permeablized using proteinase K, treated with reverse transcriptase and the resultant IL10 DNA amplified using the same primers as noted above using methodology

10 according to Bagasra, O., *et al.*, *J. Immunol. Meth.* 1993 *158*:131-145.

Cytokine mRNA in Inflamed, Post-vaccine biopsies - While metastatic melanoma is characterized by a paucity of lymphocytic infiltration (Elder *et al.*, "The surgical

15 pathology of cutaneous malignant melanoma." In: W.H. Clark, Jr., *et al.* (Eds.), *Human Malignant Melanoma*, pp. 100, New York: Grune and Stratton 1979), administration of DNP-vaccine induces T cells infiltration in metastatic masses (Berd *et al.*, 1991 *supra*.) Eight (8) subcutaneous

20 metastases (from 4 patients) that had developed inflammation following vaccine treatment were studied and compared with 3 subcutaneous metastases excised before vaccine and 4 post-vaccine metastases that failed to develop an inflammatory response. Post-vaccine, inflamed biopsies contained mRNA

25 for IFNγ (5/8), IL4 (4/8) or both (3/8). In contrast, neither IFNγ mRNA nor IL4 mRNA was detected in the 7 control specimens. All but one of these 15 tissues expressed mRNA

- 72 -

for IL10. Figure 14 shows cytokine mRNA expression for a
representative, T cell-infiltrated post-vaccine biopsy along
with the corresponding histology.

Lymph Node Metastases - A group of biopsies of melanoma

5 lymph node metastases was studied as well. Histologically,
these lesions are characterized by an abundance of
lymphocytes (Figure 15B) that are thought to be the residua
of the tumor-infiltrated lymph node lymphocytes (Cardi, et
al., Cancer Res. 1989 49:6562-6565). Of the 10 lymph node

10 biopsies studied, only one expressed mRNA for IFNγ; this
specimen, and one additional specimen, contained mRNA for
IL4. However, all 10 specimens contained mRNA for IL10.
Figure 15 shows cytokine mRNA expression for a
representative lymph node metastasis along with the

15 corresponding histology.

IL10 Production by Melanoma Metastasis and Cell Lines - As
indicated above, IL10 mRNA expression was seen in 24/25
melanoma metastases (Figure 16). Since it was independent
of IFNγ or IL4 mRNA expression and did not correlate with T

20 cell infiltration, melanoma cells, rather than lymphocytes,
may be the source of IL10. Two approaches were used to test
this hypothesis. First, cell lines derived from two of the
metastatic tumors described above were examined. As
illustrated in Figure 17A, both the cell lines and the

25 tissue from which they were derived expressed IL10 mRNA.

- 73 -

Both cell lines produced IL10, as determined by assay of culture supernatants after a 72 hour incubation (IL10 concentrations: 760 pg/ml and 10 pg/ml, respectively). Second, IL10 mRNA expression on a tissue section of a

5   melanoma metastasis was studied using *in situ* RT-PCR. As seen in Figure 17B, IL10 mRNA is associated in melanoma cells and not in non-tumor elements.

<u>TNF mRNA is expressed in melanoma metastasis</u> - mRNA for TNF in human colon carcinoma biopsies using *in situ*

10   hybridization (Naylor, M.S., et al., *Cancer Res.* 1990 *50*:4436-4440), and that resistance to TNF is associated with *in vivo* tumor growth (Lattime, E.C. and Stutman, O., *J. Immunol.* 1989 *143*:4317-4323). TNF mRNA was detected in 6/23 melanoma specimens. There was an association with DNP-

15   vaccine-induced inflammation: 4/7 T cell-infiltrated post-vaccine biopsies were positive versus 2/16 pre-vaccine or non-infiltrated post-vaccine specimens.

Example 8

This example discloses dinitrophenyl modified

20   tumor peptides for cancer immunotherapy.

Epstein barr virus (EBV) was added to B lymphoblastoid cells in culture. The B lymphoblastoid cells were transformed into a B cell tumor from the patient's own lymphocytes. Melanoma from a metastasis was cultured in

25   RPMI 1640 + 10% fetal calf serum or 10% pooled human serum. The non-adhered cells were washed off with RPMI medium.

- 74 -

When the cells were confluent, they were detached with 0.1%
EDTA and passaged into two flasks. This process continued
for about 10 to about 30 passages. To test for gamma
interferon production by T cells, lymphocytes from a

5 patient's blood were obtained. About 1,000,000 lymphocytes
were mixed with DNP modified autologous melanoma cells to
stimulate T cells. Every seven days, 100 U/ml of
interleukin-2 was added. The T cells were expanded by
passage as disclosed above. The T cells were then

10 restimulated by the DNP modified autologous melanoma cells.
An enriched population of T cells resulted which were
responsive to the DNP modified autologous melanoma cells.
Stimulation was determined by the amount of gamma interferon
production by the T cells. Generally the production of

15 gamma interferon at greater than 15 picograms/ml was
considered. These T cells were then used to test the
peptide.

Small peptides were extracted from 4 types of
cells, all generated from a single patient: 1) B

20 lymphoblastoid cells, 2) B lymphoblastoid cells modified
with dinitrophenyl (DNP), 3) cultured melanoma cells, 4)
cultured melanoma cells modified with DNP. Cells were
suspended in 0.1% trifluoroacetic acid, dounced, sonicated,
and centrifuged at 100,000 xg for 90 minutes. Material in

25 the supernatant of molecular weight >10,000 was removed by a
Centricon 10 filter. The remaining material was separated
on a reversed phase HPLC column. Individual fractions were
collected, dried, resuspended in culture medium, and added

- 75 -

to autologous B lymphoblastoid cells, which bound and
presented the peptides. These peptide-pulsed B cells were
tested for ability to stimulate a T lymphocyte cell line
that was specifically sensitized to autologous DNP-modified
5 melanoma cells.

Initially the 50 HPLC fractions (10 μl of each
sample) were pooled into five groups of ten fractions each.
As shown in Figure 13, only peptides derived from DNP-
modified melanoma cells (DNP-MEL) or DNP-modified B cells
10 (DNP-LY) were stimulatory, and only pool #2 was positive.

Each of the individual fractions of pool #2 were
analyzed by performing the T cell stimulation test with each
fraction in pool 2; activity was found only in fractions #17
and #18, and DNP-MEL peptide stimulated two-fold more gamma
15 interferon production than DNP-LY.

These results indicate that a single HPLC fraction
of low molecular weight peptide preparation contains the
peptide or peptides responsible for stimulation of T cells
sensitized to DNP modified melanoma cells.

20 Example 9

This example will determine peptide stimulation
inhibition by anti-DNP antibody.

The experiment will be identical to that described
for Example 8 with one exception. After adding peptide to
25 the B lymphoblastoid cells, and just before adding the
responding T cells, varying concentrations (1-100 μg/ml) of
anti-DNP antibody will be added to different samples. The

- 76 -

anti-DNP antibody may be obtained from the ATCC, hybridoma #CRL-1968, or a similar antibody. If the stimulation is caused by DNP modified peptides, the antibody will inhibit it. It is expected that fractions 17 and 18 will be

5 inhibited by the antibody.

Example 10

        Example 10 is expected to determine whether the responding T cells are CD4+ or CD8+.

        The experiment will be identical to that described

10 for Example 8 with one exception. The responding T cells will be fractionated into subsets before being added to the peptide-pulsed B lymphoblastoid cells. This is accomplished by mixing the T cells with magnetic beads coated with either anti-CD4 or anti-CD8 antibodies (obtained commercially from

15 Immunotech, Inc., Westbrook, Maine). Then the beads, and the cells that have bound to them are removed with a magnet. The non-binding cells are washed in tissue culture medium (RPMI + 10% pooled human serum), counted, and added to the microtiter wells for measurement of stimulation.

20 Example 11

        Example 11 discloses dinitrophenyl-modified tumor membranes for cancer immunotherapy.

        Membranes from cultured melanoma cells from one patient have been prepared according to the method of Heike

25 et al., J. Immunotherapy 1994 15:165-174, the disclosure of which is incorporated herein by reference in its entirety.

The melanoma cells were conjugated to dinitrophenyl (DNP) according to the methods of Miller and Claman, *J. Immunology* 1976 *117*:1519-1526, the disclosure of which is incorporated by reference, in its entirety. The cells were suspended in

5 5 volumes of 30 mM sodium bicarbonate buffer with 1 mM phenyl methyl sulfonyl fluoride and disrupted with a glass homogenizer. Residual intact cells and nuclei were removed by centrifugation at 1000 g. Then the membranes were pelleted by centrifugation at 100,000 g for 90 minutes. The

10 membranes were resuspended in 8% sucrose and frozen at -80° until needed. Melanoma cells were similarly prepared for conjugation to dinitrophenyl.

These DNP modified melanoma cell membranes were tested for their ability to stimulate autologous T

15 lymphocytes that had been sensitized to DNP modified intact melanoma cells. This was done by incubating about 100,000 T lymphocytes/well with about 10,000-about 100,000 cell equivalents DNP modified membranes/well, and measuring production of gamma interferon production (greater than 15

20 picograms). This process was repeated by incubating the T lymphocytes with DNP modified melanoma cells. The results revealed that intact melanoma cells and the membranes derived from them were equally effective in stimulating T cells.

25 This experiment, which has been repeated several times (using the same patient sample) with similar results, indicates that DNP modified melanoma membranes can

substitute for DNP modified intact melanoma cells in
inducing a T cell response.

Example 12

Example 12 will determine if addition of

5 autologous monocytes or dendritic cells augments the T cell
response to tumor membranes.

Autologous monocytes will be isolated as follows.
Peripheral blood lymphocytes will be separated from
peripheral blood by gradient centrifugation according to the

10 methods of Boyum, A., *Scand. J. Clin. Lab. Invest. 21*, 1968
Suppl 97:77-89, the disclosure of which is incorporated
herein by reference in its entirety. They will be suspended
in tissue culture medium (RPMI-1640 + 10% pooled human
serum) and added to plastic microtiter wells for about two

15 hours in order for monocytes to adhere. Then the non-
adherent cells will be washed off with culture medium.
Various concentrations of GM-CSF (granulocyte macrophage
colony stimulating factor, obtained commercially from
Immunex, Seattle, WA) will be added to stimulate growth of

20 the monocytes. After about 2-3 weeks, the monocytes, now
considered macrophages, will be removed from the plastic
with 0.1% EDTA and added in graded numbers (about 100 to
about 10,000/well) to fresh microtiter wells. Graded
numbers of membranes, prepared from DNP modified autologous

25 tumor cells (quantified as cell equivalents), will be added
to the adherent macrophage monolayer. After about 6 to
about 24 hours, DNP specific autologous T cells will be

added and incubated for an additional 24 hours. Then
supernatants will be collected and tested for production of
cytokines such as and not limited to gamma interferon, IL2,
tumor necrosis factor; or for proliferation or stimulation
5   of T cells such as by ^{125}IUDR, ^{3}H thymidine, or with dyes such
as MTT. For example, ^{125}IUDR will be added and the cells will
be collected on an automatic harvesting device to test for T
cell proliferation. Controls will consist of unstimulated T
cells and T cells stimulated with membranes in the absence
10 of macrophages. The ability of autologous dendritic cells
to enhance the response to membranes will be tested in the
same manner. Dendritic cells will be isolated from
peripheral blood mononuclear cells and grown in tissue
culture according to the method of O'Doherty, U., et al., J.
15 Exp. Med. 1993 178:10678-1078, the disclosure of which is
incorporated herein by reference in its entirety.

Example 13
        Example 13 is expected to determine whether
patients who received DNP modified melanoma vaccine manifest
20 delayed type hypersensitivity (DTH) to autologous DNP-
modified melanoma membranes.
        The study subjects will be patients who have
received repeated doses of DNP modified melanoma cell
vaccine. Membranes will be prepared from autologous DNP
25 modified melanoma cells as described above. Graded numbers
of membranes (about 100 to about 10,000 cell equivalents)
will be washed in PBS, resuspended in PBS, and injected

intradermally on the forearm. DTH will be measured about 48

hours later as the diameter of cutaneous induration.

Controls will consist of autologous unconjugated melanoma

cell membranes and membranes prepared from autologous blood

5 lymphocytes.

Example 14

      Example 14 is expected to determine whether

macrophages or dendritic cells process allogeneic melanoma

10 membranes and present them in an immunogenic manner to T

cells syngeneic to those macrophages.

      The procedure will be similar to that described

for Example 12 with the exception that the stimulating

membranes will be prepared from allogeneic DNP conjugated

15 melanoma cells. The hypothesis is that macrophages or

dendritic cells from patient A can *in vitro* process

membranes obtained from the melanoma cells of patient B.

This results in stimulation of T cells from patient A. This

experiment may lead to a strategy for allogeneic

20 immunization. DNP modified membranes prepared from a single

allogeneic melanoma cell line, or pool of allogeneic cell

lines, would be processed by a patient's macrophages or

dendritic cells *in vitro*. Those cells would be used for

immunization.

The disclosures of each patent, patent application and publication cited or described in this document are hereby incorporated herein by reference, in their entirety.

Various modifications of the invention in addition
5 to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Berd, David;
             Eisenlohr, Lawrence; and
             Lattime, Edmund

    (ii) TITLE OF INVENTION:
COMPOSITION COMPRISING A TUMOR CELL EXTRACT AND METHOD OF
USING THE COMPOSITION

    (iii) NUMBER OF SEQUENCES: 10
    (iv) CORRESPONDENCE ADDRESS:
      (A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz &
Norris
      (B) STREET: One Liberty Place - 46th Floor
      (C) CITY: Philadelphia
      (D) STATE: PA
      (E) COUNTRY: USA
      (F) ZIP: 19103
    (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE
      (B) COMPUTER: IBM PS/2
      (C) OPERATING SYSTEM: PC-DOS
      (D) SOFTWARE: WORDPERFECT 5.1
    (vi) CURRENT APPLICATION DATA:
      (A) APPLICATION NUMBER: Unknown
      (B) FILING DATE: June 7, 1995
      (C) CLASSIFICATION: Unknown
    (vii) PRIOR APPLICATION DATA:
      (A) APPLICATION NUMBER: 08/203,004
      (B) FILING DATE: February 28, 1994
    (viii) ATTORNEY/AGENT INFORMATION:
      (A) NAME: Lori Y. Beardell
      (B) REGISTRATION NUMBER: 34,293
      (C) REFERENCE/DOCKET NUMBER: TJU-1582
    (ix) TELECOMMUNICATION INFORMATION:
      (A) TELEPHONE: (215) 568-3100
      (B) TELEFAX: (215) 568-3439

(2) INFORMATION FOR SEQ ID NO: 1:
    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 21 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

    (iii) HYPOTHETICAL: No

    (iv) ANTI-SENSE: No

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
ATGGATGATG ATATCGCCGC G
                    21

- 83 -

(3) INFORMATION FOR SEQ ID NO: 2:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

    (iii) HYPOTHETICAL: No

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
CTAGAAGCAT TTGCGGTGGA CGATGGAGGG GCC   33

(4) INFORMATION FOR SEQ ID NO: 3:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

    (iii) HYPOTHETICAL: No

    (iv) ANTI-SENSE: No

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
ATGAAATATA CAAGTTATAT C   21

(5) INFORMATION FOR SEQ ID NO: 4:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

    (iii) HYPOTHETICAL: No

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
TTACTGGGAT GCTCTTCGAC CTCGAAACAG CAT   33

(6) INFORMATION FOR SEQ ID NO: 5:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

- 84 -

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
ATGGGTCTCA CCTCCCAACT G   21

(7) INFORMATION FOR SEQ ID NO: 6:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

    (iii) HYPOTHETICAL: No

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
TCAGCTCGAA CACTTTGAAT ATTTCTCTCT CAT   33

(8) INFORMATION FOR SEQ ID NO: 7:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

    (iii) HYPOTHETICAL: No

    (iv) ANTI-SENSE: No

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAGCTGAGAA CCAAGACCCA GACATCAAGG CG   32

(9) INFORMATION FOR SEQ ID NO: 8:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

    (iii) HYPOTHETICAL: No

    (iv) ANTI-SENSE: Yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
AGCTATCCCA GAGCCCCAGA TCCGATTTTG G   31

(10) INFORMATION FOR SEQ ID NO: 9:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

    (iii) HYPOTHETICAL: No

    (iv) ANTI-SENSE: No

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
ATGAGCACTG AAAGCATGAT C   21

(11) INFORMATION FOR SEQ ID NO: 10:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: nucleic acids

    (iii) HYPOTHETICAL: No

    (iv) ANTI-SENSE: No

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
TCACAGGGCA ATGATCCCAA AGT   23

- 86 -

What is claimed is:

1.   A composition comprising a therapeutically
effective amount of a hapten modified tumor cell extract,
said composition stimulates T cell lymphocytes and is
5 useful for the treatment of cancer.

2.   The composition of claim 1 wherein said
tumor cell extract is selected from the group consisting of
a hapten modified cancer cell membrane, a low molecular
weight peptide from a hapten modified cancer cell, an
10 antigen presenting cell with a low molecular weight peptide
from a hapten modified cancer cell bound thereto, and an
antigen presenting cell with a hapten modified cancer cell
membrane bound thereto.

3.   The composition of claim 1 wherein said
15 tumor cell extract is a low molecular weight peptide from a
dinitrophenyl modified cancer cell.

4.   The composition of claim 1 wherein said
tumor cell extract is a membrane of a dinitrophenyl
modified cancer cell.

20   5.   A composition of claim 1 wherein said tumor
cell extract is an antigen presenting cell with a low
molecular weight peptide from a hapten modified cancer
cell, or a hapten modified cancer cell membrane, bound
thereto.

25   6.   The composition of claim 1 wherein said
tumor cell extract is selected from the group consisting of
an autologous cell and an allogenic cell.

7.   The composition of claim 1 wherein said
tumor is selected from the group consisting of melanoma,
30 breast, lung, colon, breast, kidney, and prostate.

- 87 -

8.   The composition of claim 1 wherein said tumor is melanoma.

9.   The composition of claim 2 wherein said hapten is selected from the group consisting of
5 dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5 sulfonic 1-naphtyl) ethylene diamine.

10.   The composition of claim 9 wherein said hapten is dinitrophenyl.

11.   The composition of claim 1 further
10 comprising an immunological adjuvant.

12.   The composition of claim 11 wherein said immunological adjuvant is *Bacillus Calmette-Guerin*.

13.   A composition comprising a therapeutically effective amount of a dinitrophenyl modified cancer cell
15 membrane, said composition stimulates T cell lymphocytes and is useful for the treatment of cancer.

14.   A composition comprising a therapeutically effective amount of a low molecular weight peptide from a dinitrophenyl modified cancer cell, said composition
20 stimulates T cell lymphocytes and is useful for the treatment of cancer.

15.   A method for treating cancer comprising administering to a patient a therapeutically effective amount of cyclophosphamide; administering a therapeutically
25 effective amount of a hapten modified tumor cell extract wherein said extract stimulates T cell lymphocytes.

16.   The method of claim 15 wherein said tumor is selected from melanoma, lung, colon, breast, kidney, and prostate.

17. The method of claim 15 useful for the treatment of cancer selected from the group consisting of melanoma, lung cancer, colon cancer, breast cancer, kidney cancer, and prostate cancer.

5   18. The method of claim 15 wherein said tumor cell extract is selected from the group consisting of a hapten modified cancer cell membrane and a low molecular weight peptide from a hapten modified cancer cell.

19. The method of claim 18 wherein said hapten 10 is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine.

20. The method of claim 18 wherein said hapten is dinitrophenyl.

15   21. The method of claim 15 wherein said tumor cell extract is a low molecular weight peptide from a dinitrophenyl modified cancer cell.

22. The composition of claim 15 wherein said tumor cell extract is a membrane of a dinitrophenyl 20 modified cancer cell.

23. The method of claim 15 wherein said tumor cell extract is selected from the group consisting of an autologous cell and an allogenic cell.

24. The method of claim 15 wherein said 25 therapeutically effective amount of cyclophosphamide comprises administering a dose of about 300 mg/M^2 of cyclophosphamide prior to administration of said composition.

- 89 -

25. The method of claim 15 wherein said composition is mixed with said immunological adjuvant prior to administration.

26. The method of claim 25 wherein said
5 immunological adjuvant is *Bacillus Calmette-Guerin*.

27. The method of claim 15 further comprising sensitizing the patient with a therapeutically effective amount of 1-fluoro-2,4-dinitrobenzene prior to administering cyclophosphamide.

10   28. A method for the treatment of human cancer comprising administering to a patient with a therapeutically effective amount of cyclophosphamide; administering a therapeutically effective amount of a tumor cell extract which stimulates T cell lymphocytes, said
15 composition mixed with an immunological adjuvant; and administering a therapeutically effective amount of a cytokine selected from the group consisting of interleukin-12, interleukin-2, and interleukin-13.

29. The method of claim 28 wherein said tumor
20 cell extract is selected from the group consisting of a hapten modified cancer cell membrane and a low molecular weight peptide from a hapten modified cancer cell.

30. The method of claim 29 wherein said hapten is selected from the group consisting of dinitrophenyl,
25 trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine.

31. The method of claim 29 wherein said hapten is dinitrophenyl.

32. The method of claim 28 wherein said tumor cell extract is a low molecular weight peptide from a dinitrophenyl modified cancer cell.

33. The composition of claim 28 wherein said
5 tumor cell extract is a membrane of a dinitrophenyl modified cancer cell.

34. The method of claim 28 wherein said tumor cell extract is selected from the group consisting of an autologous cell and an allogenic cell.

10   35. The method of claim 28 wherein said therapeutically effective amount of cyclophosphamide comprises administering a dose of about 300 mg/M^2 of cyclophosphamide prior to administration of said composition.

15   36. The method of claim 28 wherein said composition is mixed with an immunological adjuvant prior to administration.

37. The method of claim 28 wherein said immunological adjuvant is *Bacillus Calmette-Guerin*.

20   38. The method of claim 28 wherein said tumor is selected from melanoma, lung, colon, breast, kidney, and prostate.

39. The method of claim 28 useful for the treatment of cancer selected from the group consisting of
25 melanoma, lung cancer, colon cancer, breast cancer, kidney cancer, and prostate cancer.

40. The method of claim 28 further comprising sensitizing the patient with 1-fluoro-2,4-dinitrobenzene prior to administrating cyclophosphamide.

41. A method for treating cancer comprising administering to a patient a therapeutically effective amount of cyclophosphamide; administering a therapeutically effective amount of a tumor cell extract which stimulates T
5 cell lymphocytes, said composition mixed with an immunological adjuvant; administering a therapeutically effective amount of a non-haptenized, irradiated composition comprising a tumor cell extract.

42. A method of screening for cytokine
10 production by tumors to determine the efficacy of an autologous, irradiated hapten conjugated cell composition to alleviate cancer in a patient suspected of having cancer, said method comprising:
    administering said hapten conjugated composition
15 to said patient;
    obtaining a sample comprising nucleic acids from a patient tissue sample;
    amplifying nucleic acids specific for a cytokine or amplifying a signal generated by hybridization of a
20 probe specific to a cytokine specific nucleic acid in said tissue sample; and
    detecting the presence of the amplified nucleic acids or the amplified signal, wherein the presence of amplified nucleic acids or amplified signal from said
25 patient tissue sample indicates cytokine production and determines efficacy of said hapten conjugated composition.

43. The method of claim 42 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl)
30 ethylene diamine.

44. The method of claim 42 wherein said patient tissue sample is a subcutaneous inflammation.

- 92 -

45. The method of claim 42 wherein said amplification step comprises hybridization to a cytokine specific nucleic acid of at least one oligonucleotide which is complementary to a cytokine specific sequence.

5   46. The method of claim 42 wherein the nucleic acids specific for a cytokine comprise nucleic acids encoding gamma interferon, tumor necrosis factor, interleukin 2, interleukin 12, and interleukin 13.

47. The method of claim 42 wherein said
10 amplification step comprises hybridization to a cytokine specific nucleic acid with a pair of primers, wherein one primer within said pair is complementary to cytokine specific sequence.

48. The method of claim 42 wherein said
15 amplification step comprises performing a procedure selected from the group consisting of polymerase chain reaction, ligase chain reaction, repair chain reaction, cyclic probe reaction, nucleic acid sequence based amplification, strand displacement amplification, and Qβ
20 replicase.

49. The method of claim 42 wherein said amplification step comprises performing a polymerase chain reaction, wherein said polymerase chain reaction comprises a first primer and a second primer, wherein said first
25 primer is selected from the group consisting of SEQUENCE ID NOS: 1, 3, 5, 7, and 9, and said second primer is selected from the group consisting of SEQUENCE ID NOS: 2, 4, 6, 8, and 10.

- 93 -

50. The method of claim 42 wherein said
amplification step comprises performing a polymerase chain
reaction wherein said polymerase chain reaction comprises a
first primer and a second primer, wherein said first primer
5 is SEQ ID NO: 3 and said second primer is SEQ ID NO: 4.

51. The method of claim 42 wherein said
amplification step comprises performing a polymerase chain
reaction, wherein said polymerase chain reaction comprises
a pair of primers, wherein one primer of said pair is
10 complementary to a cytokine specific sequence.

52. The method of claim 42 wherein the primer
that is complementary to a cytokine specific sequence is
selected from the group consisting of SEQUENCE ID NOS: 1 to
10.

15   53. The method of claim 47 wherein the primer
that is complementary to cytokine specific sequence is
selected from the group consisting of SEQ ID NOS: 1 to 10.

54. The method of claim 47 wherein the primer
that is complementary to a cytokine specific sequence is
20 SEQ ID NO: 3.

55. The method of claim 42 wherein said patient
tissue sample is a tissue selected from the group
consisting of a tumor, saliva, sputum, mucus, bone marrow,
serum, blood, urine, lymph, and a tear.

25   56. A diagnostic kit for screening for the
efficacy of an autologous, irradiated, hapten conjugated
cell composition comprising in one or more containers, a
pair of primers, wherein one of the primers within said
pair is complementary to a cytokine specific sequence,
30 wherein said primer is selected from the group consisting
of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4,

- 94 -

SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ
ID NO: 9, and SEQ ID NO: 10, and a means for visualizing
amplified DNA; said kit useful for determining the efficacy
of said composition.

5   57.   The diagnostic kit of claim 56 wherein said
means for visualizing amplified DNA is selected from the
group consisting of ethidium bromide stain, ^{32}P, and biotin.

        58.   The method of claim 42 wherein said patient
tissue sample is a melanoma tissue.

10   59.   A method of screening for cytokine
production by a tumor to determine the efficacy of an
autologous, irradiated hapten conjugated cell composition
in a patient suspected of having cancer, said method
comprising:

15   obtaining a sample of RNA from a patient tumor
sample;

        reverse transcribing said RNA into DNA;

        amplifying said DNA with polymerase chain
reaction using a pair of primers which are complementary to
20 separate regions of a cytokine sequence; and

        detecting the presence or absence of amplified
DNA wherein the presence of amplified DNA indicates
cytokine production and determines efficacy of said hapten
conjugated composition.

25   60.   The method of claim 59 wherein the
polymerase chain reaction is *in situ* polymerase chain
reaction.

AMENDED CLAIMS

[received by the International Bureau on 18 November 1996 (18.11.96);
new claims 61-80 added;
remaining claims unchanged (2 pages)]

61. A composition comprising a hapten modified human tumor cell.

62. A composition of claim 61 wherein said tumor is selected from the group consisting of melanoma, lung, colon, breast, kidney, and prostate.

63. A composition of claim 61 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N' (5 sulfonic 1-naphtyl) ethylene diamine.

64. A composition of claim 61 further comprising an adjuvant.

65. A composition of claim 64 wherein said adjuvant is selected from the group consisting of *Bacillus Calmette-Guerin*, cytokines and QS-21.

66. A composition of claim 61 further comprising a carrier.

67. A composition of claim 66 wherein said carrier is selected from the group consisting of saline and culture medium.

68. A composition comprising a hapten modified human tumor cell wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N' (5 sulfonic 1-naphtyl) ethylene diamine and an adjuvant selected from the group consisting of *Bacillus Calmette-Guerin*, cytokines and QS-21.

69. A composition comprising a hapten modified human melanoma tumor cell.

70. A composition comprising a hapten modified human lung tumor cell.

71. A composition comprising a hapten modified human colon tumor cell.

72. A composition comprising a hapten modified human breast tumor cell.

73. A composition comprising a hapten modified human kidney tumor cell.

74. A composition comprising a hapten modified human prostate tumor cell.

75. A composition of claims 69-74 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N' (5 sulfonic 1-naphtyl) ethylene diamine.

76. A composition of claim 75 further comprising a carrier.

-96-

77.   A composition of claim 76 wherein said carrier is selected from the group consisting of saline and culture medium.

78.   A composition of claim 75 further comprising an adjuvant selected from the group consisting of *Bacillus Calmette-Guerin*, cytokines and QS-21.

79.   A composition of claim 78 further comprising a carrier.

80.   A composition of claim 79 wherein said carrier is selected from the group consisting of saline and culture medium.

STATEMENT UNDER ARTICLE 19

Claims 61-80 are newly added on substitute pages 94/1 and 94/2. Support for the newly added claims may be found in the specification as filed, see for example, page 16, lines 1-2; page 20, lines 11-28; page 23, lines 1-22; page 28, lines 6 and 8; page 63, line 7; page 73, line 26; page 74, line 28.

The claims are believed to be free of prior art.

FIG. 1

FIG. 2

Figure 3

FIG. 4

FIG. 5

Figure 6

7/19

FIG. 7

8/19

FIG. 8
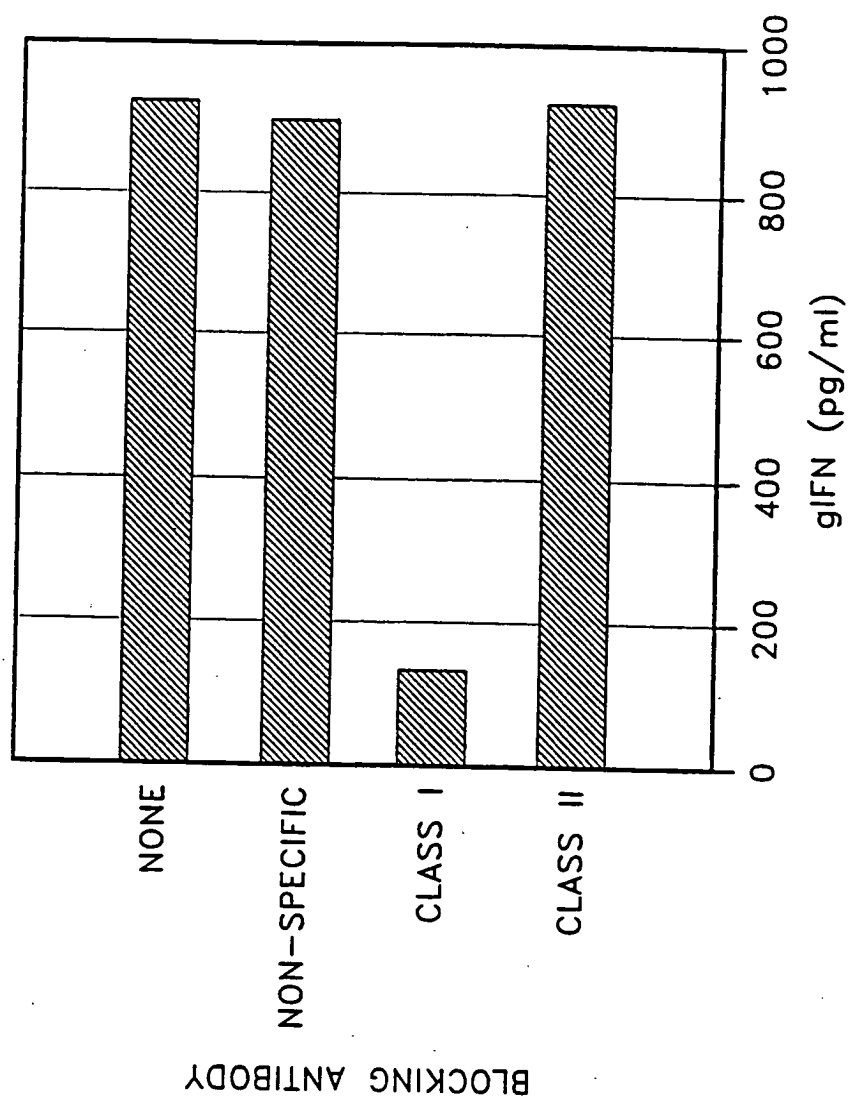
FIG. 9
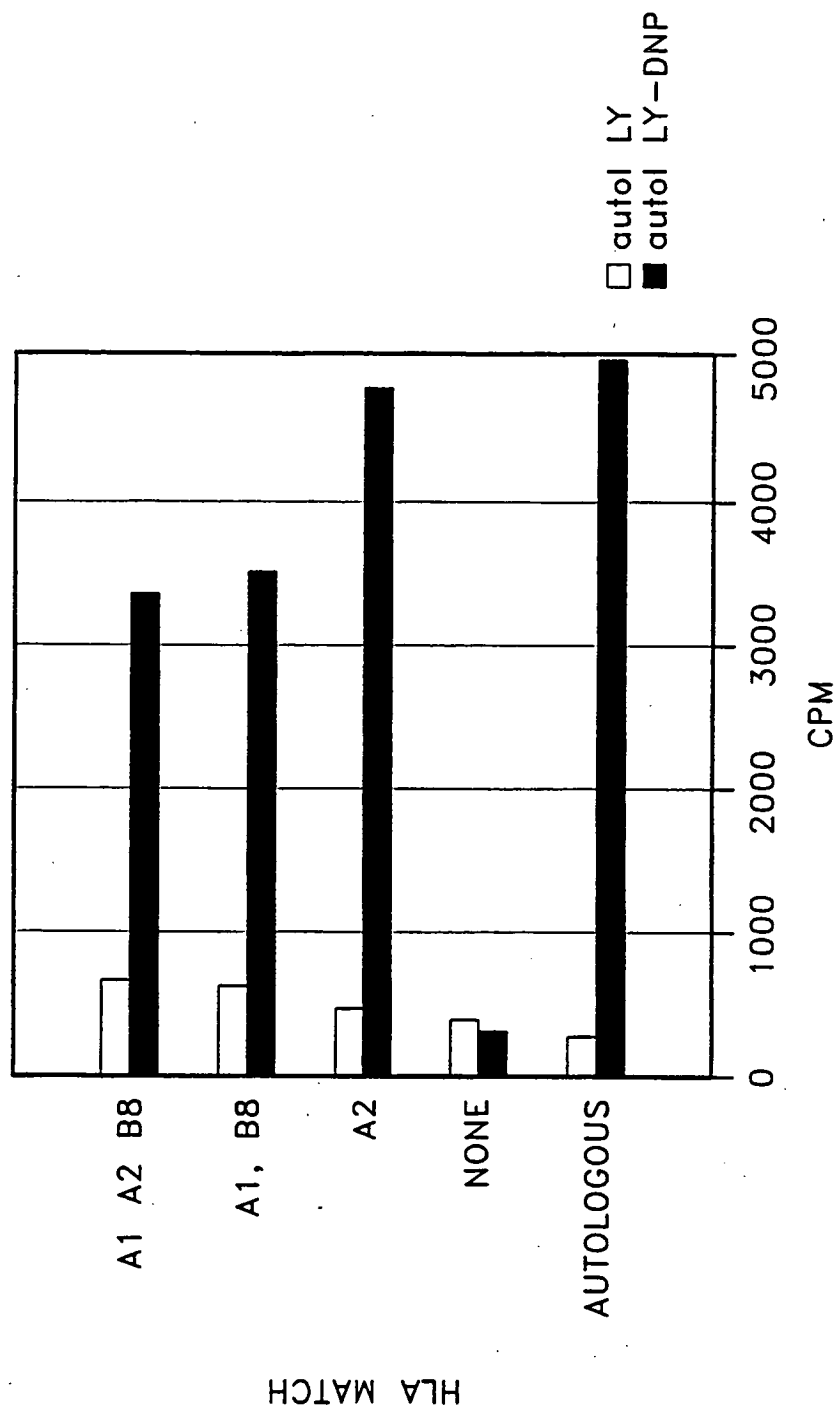
FIG. 10
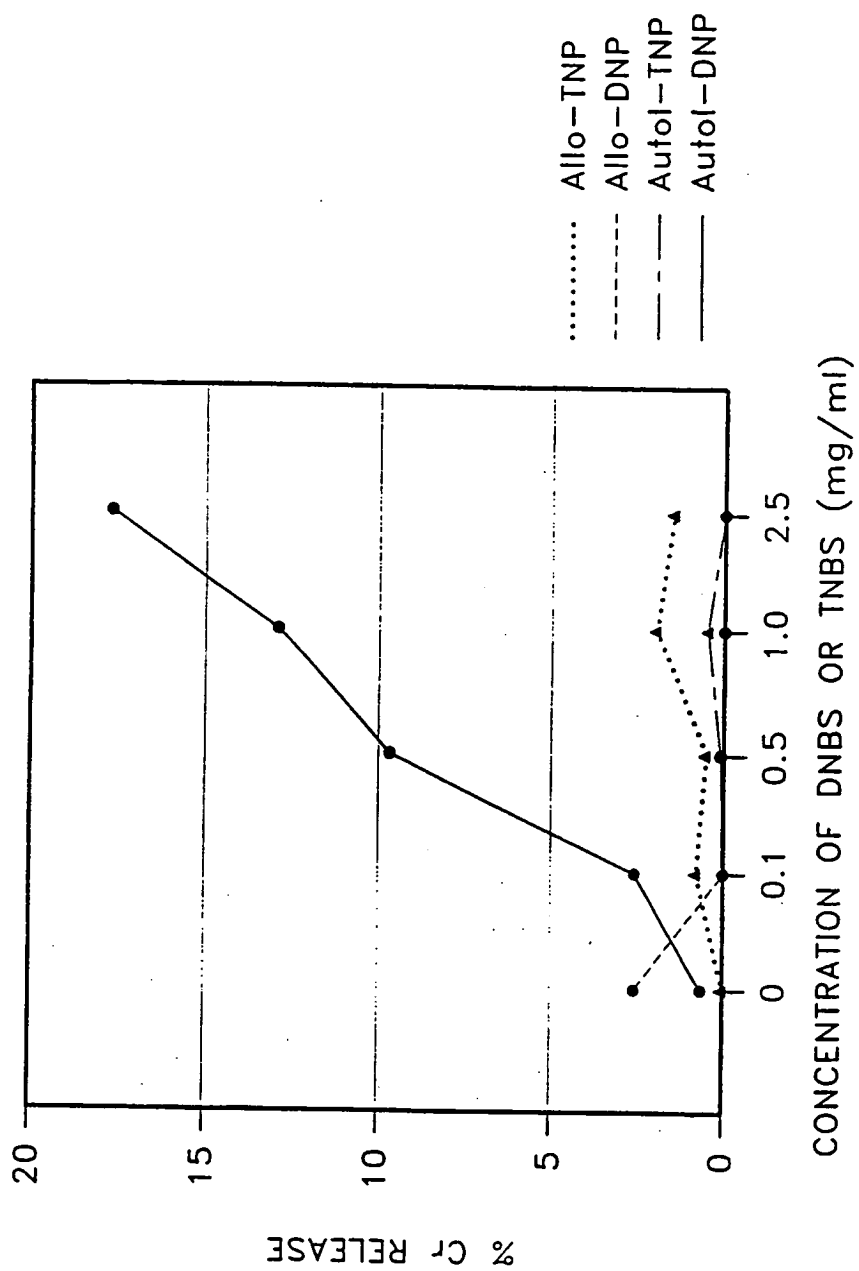
FIG. 11A

12/19
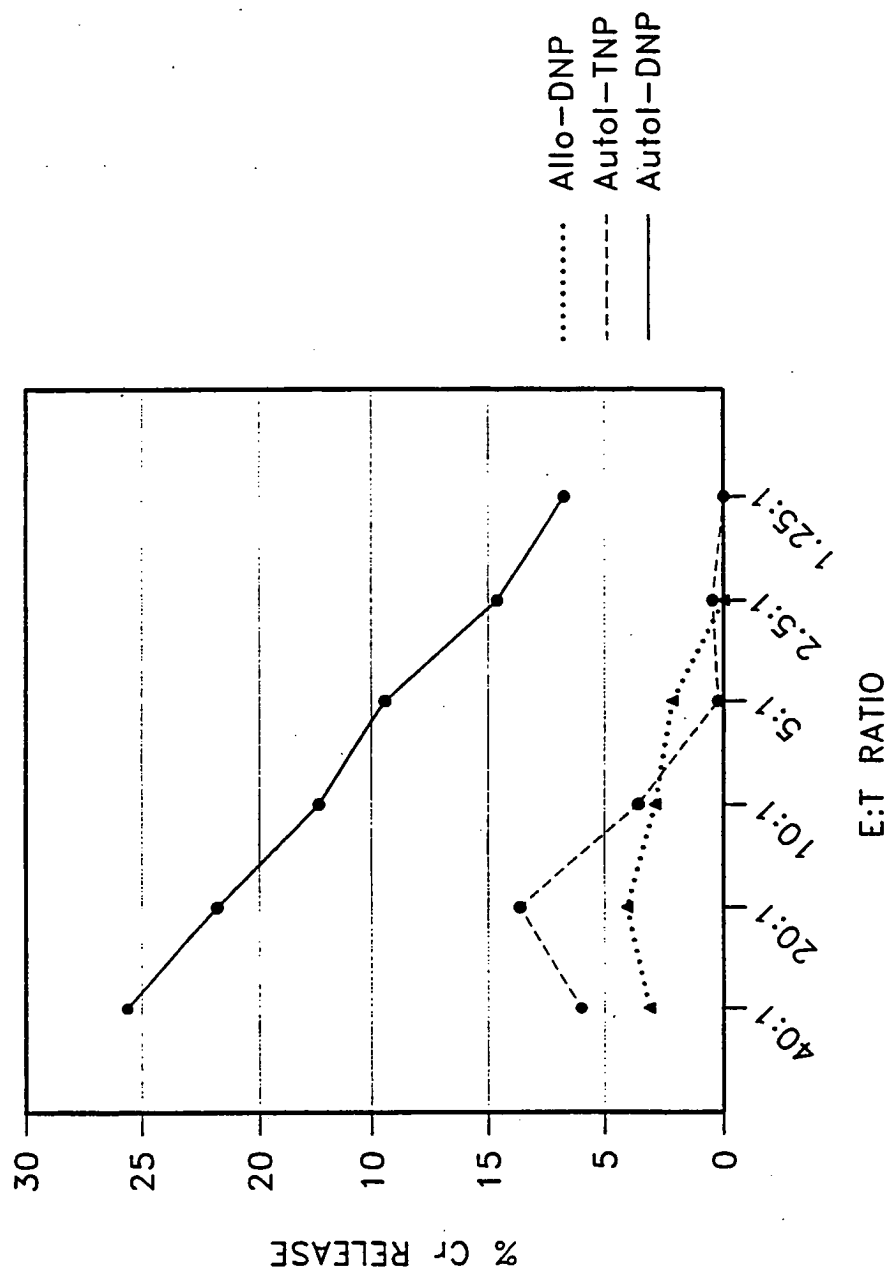
FIG. 11B

ADJUVANT TREATMENTS IN STAGE 2 MELANOMA
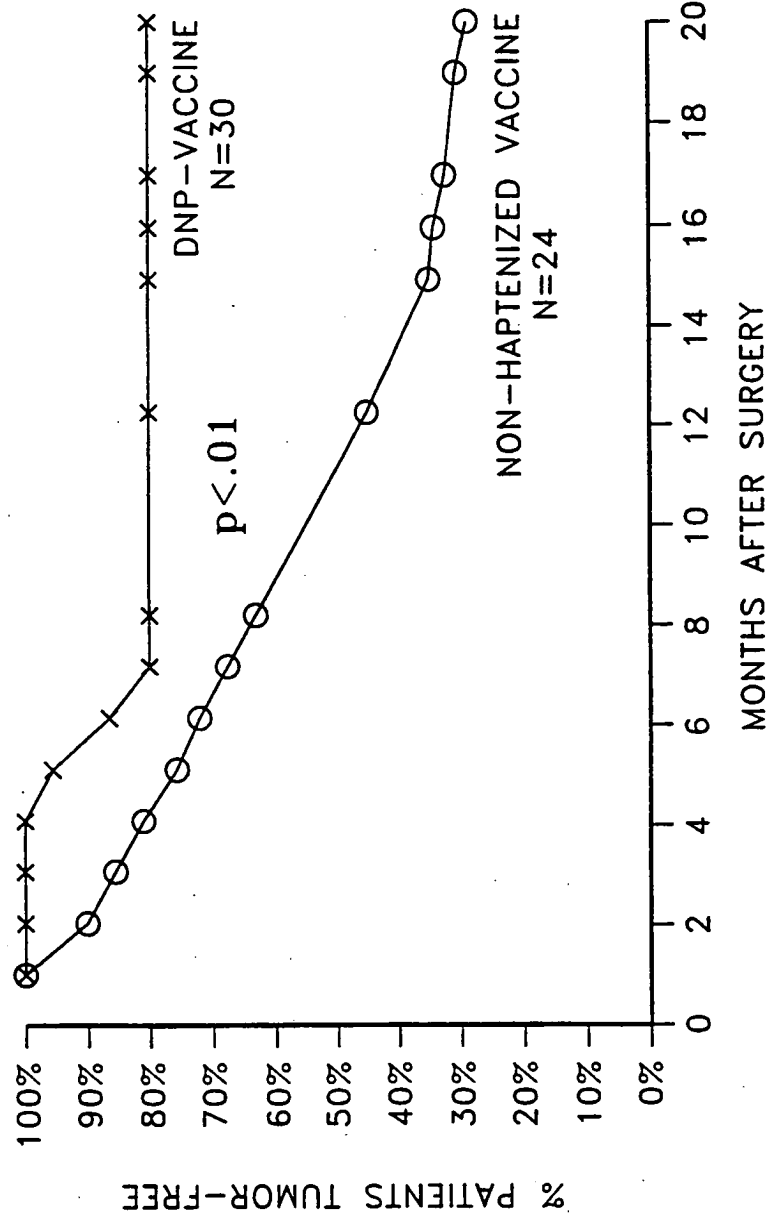
FIG. 12

14/19

**T CELL STIMULATION BY PEPTIDES
FROM DNP-MODIFIED MELANOMA CELLS**
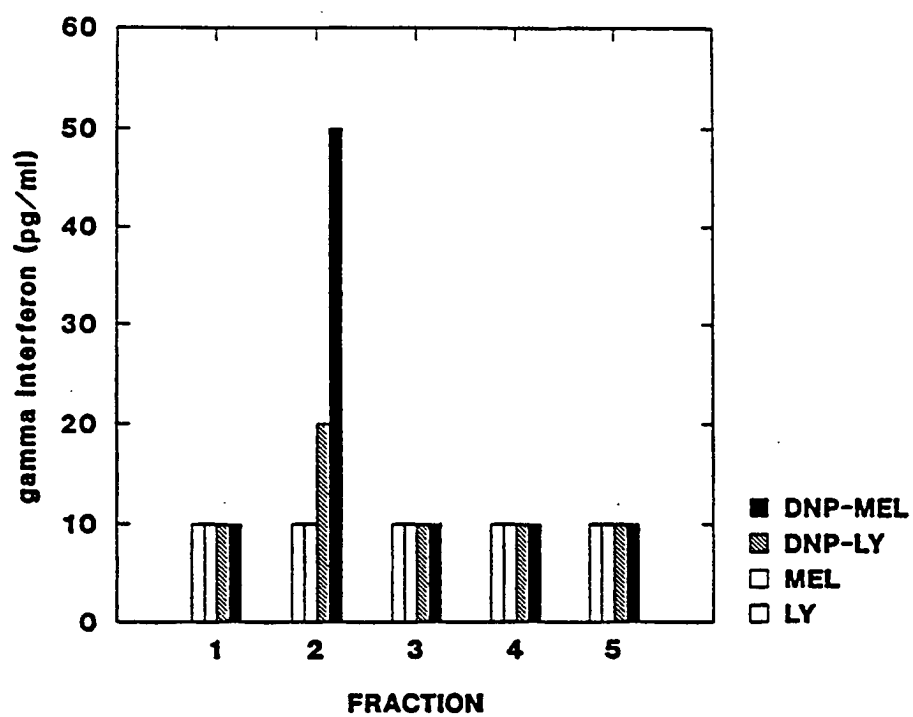
FIG. 13
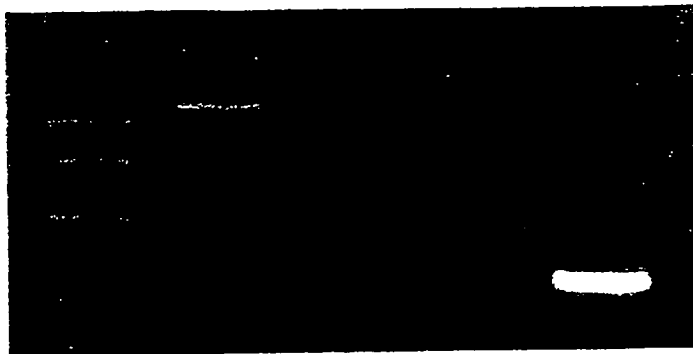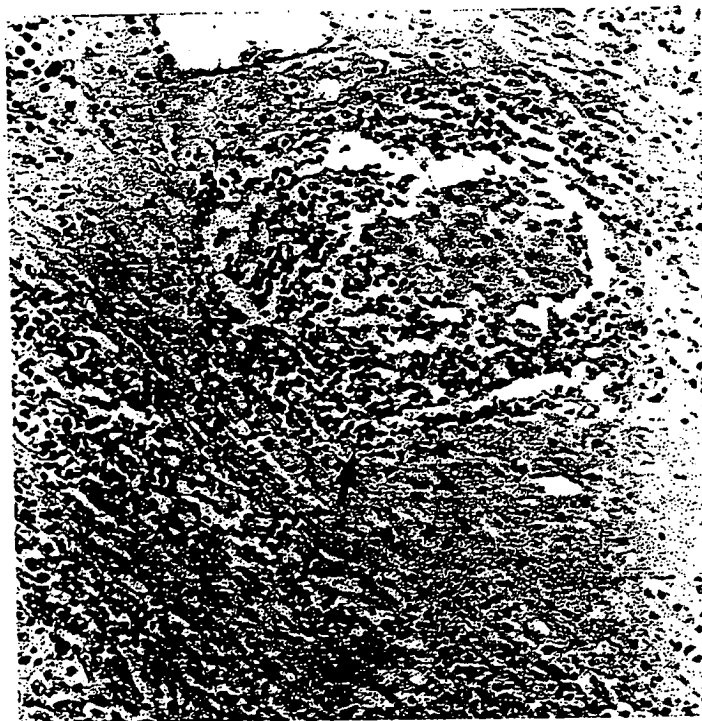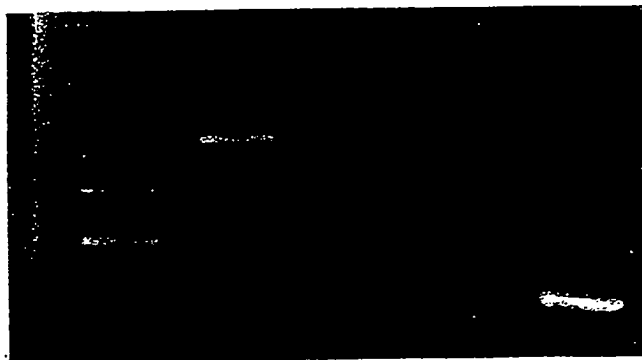
FIG. 14A

FIG. 14B

FEUILLE RECTIFIEE (REGLE 91)

FIG. 15A

FIG. 15B

Patient

C 1 2 3 4 5 6 7

IL10

C cDNA Control

FIG. 16

18/19

FIG. 17

FIG. 18A

FIG. 18B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   424/85.2, 85.4, 184.1, 185.1, 193.1, 278.1, 520; 435/6, 91.2, 961; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	SIA et al. Studies in the enhancement of tumour immunity by coupling strong antigens to tumour cells ('heterogenization of tumours'). Helper T cell clones against PPD help other T cells mount anti-tumour responses to PPD-coupled tumour cells. Immunol. April 1984, Vol. 51, pages 755-763, see entire document.	1-12 ---------- 13-40
Y	KOSUGI et al. Cross-reactivity between haptenic muramyl Di- or Tripeptide derivatives and *Mycobacterium bovis* BCG: Potential application for enhancing tumor immunity. Infection & Immunity. December 1986, Vol. 54, No. 3, pages 768-773, see entire document.	1-41

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 SEPTEMBER 1996	17 SEP 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	RAY F. EBERT
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BERD et al. Immunization with haptenized, autologous tumor cells induces inflammation of human melanoma metastases. Cancer Research. 15 May 1991, Vol. 51, pages 2731-2734, especially pages 2731-2732.	1-13 and 15-27 ---------- 14 and 28-40
X --- Y	BERD et al. Treatment of human melanoma with a hapten-modified autologous vaccine. . Ann. New York Acad. Sci. 16 September 1993, Vol. 690, pages 147-152, see entire document.	1-13 and 15-27 ---------- 14 and 28-40
Y	MACLEAN et al. Active immunization of human ovarian cancer patients against a common carcinoma (Thomsen-Friedenreich) determinant using a synthetic carbohydrate antigen. J. Immunotherapy. April 1992, Vol. 11, pages 292-305, see entire document.	1-41
Y	HELLSTROM et al. Tumor immunology: an overview. Ann. New York Acad. Sci. 16 September 1993, Vol. 690, pages 24-33, especially page 29, paragraph 5.	1-40
Y	WO 90/11764 A1 (FUNG ET AL) 18 October 1990 (18.10.90), Abstract and pages 1-3	41
Y.	WO 87/06840 A1 (SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH) 19 November 1987 (19.11.87), see entire document, especially pages 1-4.	41
X --- Y	BYSTRYN, J-C. Tumor vaccines. Cancer & Metastasis Reviews. January 1990, Vol. 9, pages 81-91, especially pages 82-83.	41 ---------- 1-40
Y	HOOVER et al. Prospectively randomized trial of adjuvant active-specific immunotherapy for human colorectal cancer. Cancer. March 1985, Vol. 55, pages 1236-1243, see entire document.	41
Y	KANANGAT et al. Use of quantitative polymerase chain reaction to quantitate cytokine messenger RNA molecules. Molec. Immunol. 1992, Vol. 29, No. 10, pages 1229-1236, see entire document.	42-60
Y	NITTA et al. Expression of tumour necrosis factor-α, -β and interferon-γ genes within human neuroglial tumour cells and brain specimens. Cytokine. March 1994, pages 171-180, see entire document.	42-60

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KAASHOEK, J.G.J. Cytokine production by the bladder carcinoma cell line 5637: Rapid analysis of mRNA expression levels using a cDNA-PCR procedure. Lymphokine & Cytokine Res. 1991, Vol. 10, No. 3, pages 231-235, see entire document.	42-60
Y	PISA et al. Selective expression of interleukin 10, interferon γ, and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies. Proc. Natl. Acad. Sci. USA. August 1992, Vol. 89, pages 7708-7712, see entire document.	42-60
X --- Y	LUSCHER et al. The pattern of cytokine gene expression in freshly excised human metastatic melanoma suggests a state of reversible anergy of tumor-infiltrating lymphocytes. Int. J. Cancer. October 1994, Vol. 57, pages 612-619, see entire document.	42-55 and 58 ---------- 56-57, 59 and 60
Y	CARDING et al. A polymerase chain reaction assay for the detection of cytokine gene expression in small numbers of cells. J. Immunol. Meth. 22 July 1992, Vol. 151, pages 277-287, see entire document.	42-60
X --- Y	GUNNING et al. Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. Mol. Cell. Biol. May 1983, pages 787-795, see entire document.	49, 50, 52-54, 56 ---------- 42-48 and 57-60
Y	WO 95/15334 A1 (CALIFORNIA PACIFIC MEDICAL CENTER RESEARCH INSTITUTE) 08 June 1995 (08.06.95), see entire document, especially cytokine sequences.	49, 52, 53, 56
Y	WO 94/21792 A2 (VIAGENE, INC.) 29 September 1994 (09.29.94) see entire document, especially cytokine sequences.	49, 50, 52-54, 56
Y	GB 2218420 A (BRITISH BIOTECHNOLOGY LIMITED) 15 November 1989 (11.15.89) see entire document, especially IL-4 sequence.	49, 50, 52-54, 56
X --- Y	VIEIRA et al. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. Proc. Natl. Acad. Sci. 15 February 1991, Vol. 88, pages 1172-1176, see entire document.	49, 50, 52-54, 56 ---------- 42-48, 51, 55 and 57-60

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09511

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

   ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 35/12, 38/00, 38/21, 39/00, 39/385, 45/00, 45/05, 47/00
A01N 37/18; C12P 19/34; C12Q 1/68; G01N 33/531

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.2, 85.4, 184.1, 185.1, 193.1, 277.1, 278.1, 520; 435/6, 91.2, 961; 514/2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, WPIDS, BIOSIS, EMBASE
Search terms:
tumor/vaccine/melanoma/hapten/bcg/cyclophosphamide/interleukin/amplif?
cytokine/expression/pcr/Berd,D

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I.   Claims 1-40, drawn to compositions and methods for treating cancer using hapten-modified tumor cell
extracts.

Group II.   Claim 41, drawn to a method of treating cancer using non-haptenized/irradiated tumor cell extracts mixed
with adjuvant.

Group III. Claims 42-60, drawn to in vitro methods of screening for cytokine production by tumors using nucleic acid
probes and amplification techniques.

The groups listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule
13.2, they lack the same or corresponding special technical features for the following reasons: The special technical
feature of Group I is hapten-modified tumor cell extracts claimed in a product and a method of treating; the special
technical feature of Group II is non-haptenized/irradiated tumor cell extracts claimed in a method of treating; the special
technical feature of Group III is a screening method based on the level of cytokine production, as determined by nucleic
acid amplification and detection. The special technical features are not so linked as to form a single inventive concept
because they differ in compositions (e.g., haptenized vs. non-haptenized cell extracts), method steps (e.g., methods of
treatment vs. methods of screening), objectives (e.g., tumor cell killing vs. tumor cell monitoring), and outcomes (e.g.,
reduction or elimination of tumor vs. detection of tumor or response to treatment). Accordingly, the claims are not so
linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.